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W. B. BRIERLEY

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C. T. GIMINGHAM

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THE VERNALIZATION OF TOMATO SEED

BY D. W. GOODALL AND B. D. BOLAS

From the Research Institute of Plant Physiology, Imperial College of Science and Technology, London, S.W.7, and the Experimental and Research Station, Cheshunt

THE term 'vernalization' ('yarovization') was introduced to describe a process by which winter cereals were made to behave as spring cereals ('yarovoye') in respect of flowering. The process developed commercially in Russia consisted in allowing the grain to germinate with such a limited supply of water that germination could not proceed beyond the first appearance of the radicle, and then chilling it for some weeks at a temperature just above freezing-point. The grain thus treated could be sown in spring, and would come into ear during the summer of the same year.

Considerable work has been devoted to an attempted analysis of vernalization in cereals; much of this has been reviewed by Whyte (1939). The two principal conclusions are: (*a*) that vernalization acts by reducing the number of leaves formed before flowering (Purvis, 1934); and (*b*) that vernalization is closely linked with photoperiodism, so that the effect of seed chilling can be modified by the length of day during subsequent development.

The success achieved in the vernalization of winter cereals has led to much experimentation to find whether similar treatment of the seeds of other plants would also lead to earlier flowering, and perhaps to a greater crop. Experiments have been carried out on other Gramineae and on various dicotyledons. Plants in respect of which success has been claimed include soybean, cotton and the tomato.

The first such experiments on the tomato known to the writers were those of McMillan *et al.* (1934). They germinated seeds on moist blotting paper for 5 days, and then subjected them to a temperature of 25° C. for 12 and 24 days. They found no difference from the controls in date of fruiting or yield.

Burr & Turner (1935) treated seeds of the variety Ailsa Craig for various periods, and sowed them after storage for some time in the laboratory. They found that the plants grew poorly, and that their flowering was delayed. In a later paper (Turner & Burr, 1937) they suggest that this was due to the partial drying of the seeds before they were sown. Drying of cereal seeds after vernalization is known to undo the effect (Gregory & Purvis, 1938). A subsequent experiment was so arranged that the seeds could be sown immediately after chilling. Although the seeds which had been chilled for 24 days were sown 24 days later than the controls, the plants they produced flowered on average 14 days earlier, bore fruit for the first time 11 days earlier, and gave a total crop nearly 10 % greater than the unvernalized plants. Subsequent work did not confirm these results, and in 1938 Turner (private communication) stated that 'the treatment. . . does not hasten the fruiting of the crop'.

Avakijan (1936) tried the effect of keeping germinated tomato seeds in moist muslin or sawdust at 8, 10–12, 20–25 and 30° C. for various periods before planting. Sixty varieties were used; many of them were favourably affected by the lower temperature treatments, others by treatment at 20–25° C., while in some no vernalization treatment was effective. Prolonging the treatment beyond 10 days was without further effect. Where the treatment had been successful flowering took place 5–6 days earlier than in the controls, the plants bore more trusses, and there was more fruit on each truss. The total crop was increased by some 30 %.

Litvinov & Lukjyanov (1938) also claim success in the vernalization of tomato seeds. Their method was to germinate seeds, allow them to dry for 20–30 min., and then to place them in Petri dishes in a saturated atmosphere, but without contact with water. These dishes of seeds were placed at temperatures of 2–3, 18–20 and 25–30° C. for 10, 15 and 20 days. Sprouted seeds without previous tem-

perature treatment were sown as controls. The varieties used in 1935 were Tuckswood and Bison; the authors state that the Bison seedlings treated at 2–3° C. grew well at first, but soon lost their initial advantage. Those treated at 18–20° C. flowered 5 days earlier than the controls, were somewhat delayed in setting, but ripened fruit 6 days in advance of the untreated plants. A vernalization period of 10–15 days is recommended. The experiments with the variety Tuckswood were inconclusive. In 1936 they continued their work, using the variety Sparks Earliana. In this case, the optimum vernalization temperature was found to be 10–12° C. An acceleration of 6 days in fruiting was obtained, and an increase in the total yield of 31 %. The optimum period of vernalization is given as 5–10 days.

Vasiljev (1939) also reports some experiments with the variety Stark. He found no effect either when sprouted seeds were subjected to a temperature of 0–2° C. for 65 days, or when seedlings were kept at 5–6° C. for 20 days, followed by a period of 15 days during which the temperature was gradually raised to 10–12° C.

The results of the investigations mentioned above should not be accepted without reserve, for only small numbers of plants were grown and the figures were not subjected to statistical analysis.

The present writers began their work on the vernalization of tomatoes early in 1938. At that time they were acquainted only with the paper of Turner & Burr (1937), who germinated the seeds (var. Ailsa Craig) on moist filter paper in Petri dishes at 24° C. for 40 hr.; by that time 5–9 % of the seeds showed signs of the radicle just appearing (Turner, private communication). They then chilled the seeds at 31·5° F. for 12 and 24 days, and sowed immediately the treatment was complete.

The variety Potentate was used in the experiments at Cheshunt. Seeds were germinated in Petri dishes until 50 % showed visible signs of germination, and they were then kept at 0° C. for 20 days. It was found that this treatment killed most of the seeds; the reason may lie in the difference of the variety from that used by Turner & Burr, or in the seeds being at a more advanced stage of development when chilling began. Accordingly less drastic chilling conditions (1 and 5° C.) were tried, and some of the seeds were chilled when they had been under germination conditions for only half the time needed for 50 % of them to germinate. The results, which have already been published (Goodall & Bolas, 1939), were inconclusive, but suggested that the treatment tended to delay and reduce fruiting. However, some plants grown from seeds left imbibed at low temperatures (0 and 1° C.) without previously having had an opportunity to germinate, and subsequently allowed to become rather dry, gave promising results; it was decided to continue on these lines.

Experimental methods

The seed used was 'Potentate, virus free'. Two refrigerators were available, the temperature of one remaining fairly constant at 7° C., while that of the other fluctuated between 2 and 3° C. In addition, an ice bath giving a temperature of 0° C. was available; and seeds placed on top of the 7° C. refrigerator could be kept at a temperature fluctuating between 8 and 11° C.

On 2 Dec. 1938, seeds were spread on well-moistened filter paper in small Petri dishes, and four such dishes were placed in each of the four temperature conditions; on 12 Dec. four more dishes were put at each temperature. On 22 Dec. all the dishes were removed from the refrigerators and placed in an incubator at 14° C., together with eight dishes of seeds not subjected to chilling. Before the dishes were placed in the incubator, each was given just so much water as would not drain from the filter paper.

The dishes were left open in the incubator and a tray of calcium chloride was put in it at the same time. Thus by 26 Dec. all the dishes had lost much of their water. On that date the calcium chloride was removed and half of each of the nine groups of dishes was remoistened and covered. On 30 Dec. the filter papers in the dishes which had been left open were almost dry, the others were still quite wet; they will be referred to respectively as the 'dry' and the 'moist' series.

On 30 Dec. almost all the seeds had germinated, and the radicles ranged up to 3 cm., being longer in the 'dry' series, and also longer in those chilled before germination than in the controls. Those chilled at 8–11° C. had the longest radicles. On that day the seedlings were planted separately with forceps in seed boxes, forty-five to each box. Seedlings damaged or abnormal in any way were rejected. Each box was sown from a separate dish; there were thus two boxes of each chilled batch, and four of each of the two unchilled batches.

A record was kept of the date at which each seedling emerged from the soil since it was thought that this might affect subsequent growth—as in the previous year's experiment. In a seed box sown in the usual way the seedlings usually emerge almost simultaneously. In the present instance, on account of the varying stages of development at which the seedlings were planted, emergence was much less regular and in most of the boxes extended over 4–5 days, in some as much as 10 days.

During the week ending 3 Feb. the seedlings were potted in '60' pots, fifteen normal specimens being selected for this purpose from each of the boxes sown with chilled seed and thirty from each of the control boxes. Between 3 Feb. and 17 Mar. the lengths of the first, third, fifth, seventh and ninth leaves were measured weekly in one-third of the plants from each seed box, in order to give some indication of the rate of vegetative development.

At the end of March the plants were ready for planting in the greenhouse, which could accommodate on each side forty-eight rows of five plants each. The house was accordingly divided into four blocks, in each of which a row was planted from each of the sixteen batches grown from chilled seed together with four rows from each of the two control batches. Within each block the treatments were randomized. The plants for each row were selected at random from those available, except that each of the seed boxes for that treatment was represented as equally as possible in each row.

The date of the opening of the first flower was recorded for each plant. Soon after flowering had commenced, it was found that a number of the plants were showing symptoms of mosaic disease. These were immediately removed, but the disease continued to spread and more plants had to be removed during the next 2 weeks. At this stage it was evident that there was no hope of eradicating the disease, and that the choice must be made between continuing with some of the diseased plants or abandoning the experiment. The former course was decided upon. By this time, 141 plants out of 480 had been removed, including seven complete rows. On analysing the figures for number of survivors it was found that there were no significant differences between treatments. There were, however, very substantial differences between blocks, one having lost 50 % of the plants, while another had lost only 10 %.

The first fruits were gathered on 2 June, and gathering continued three times a week till the end of August. At that time, however, the outbreak of war interfered with the regularity of gathering, and the last fruits were collected on 27 Sept. Unripe fruits were not gathered. For the first 3 weeks of cropping the fruit from each plant was recorded separately, but subsequently this was impracticable and the crop was recorded row by row. Both weight and number of fruits were noted, but no record was kept of the number of trusses which ripened fruits, or of how many fruits each produced.

Results

(*a*) *Time of emergence.* The seedlings from unchilled seeds emerged from the soil in the seed box on average about half a day later than those from chilled seeds (see Table 1). This difference was not significant nor were any of the other differences among the treatments. In some earlier vernalization trials results have been ascribed to the temperature treatments to which the seeds had been subjected, when in fact they were simply due to the treated seeds having been sprouted before sowing, the plants grown from them being thus at a more advanced stage throughout development than the controls (Sprague, 1934). In the present experiments, the possibility of differing dates of emergence being responsible for differences in later development was borne in mind.

(*b*) *Position of first leaves.* Goodall (1937, 1938) showed that the relative position of the first two rough leaves is affected by the previous history of the seed. In over 95 % of plants grown from fresh seeds the points of insertion of these leaves follow the same spiral phyllotaxis as the subsequent leaves. If older seeds are used, or the dry seeds have previously been

TABLE 1. *Summary of results: treatment means*

Days of chilling treatment	Drying during germination, °C.					Moist during germination, °C.					Mean, °C.				
	0	2–3	7	8–11	Mean	0	2–3	7	8–11	Mean	0	2–3	7	8–11	Mean
Days from sowing to emergence															
0	—	—	—	—	4·03	—	—	—	—	3·73	—	—	—	—	3·88
10	3·20	2·90	3·50	4·00	3·40	2·70	3·50	2·90	2·50	2·90	2·95	3·20	3·20	3·25	3·15
20	2·90	3·60	3·50	3·00	3·25	2·90	5·90	2·90	3·80	3·88	2·90	4·75	3·20	3·40	3·56
Mean	3·05	3·25	3·50	3·50	3·47	2·80	4·70	2·90	3·80	3·88	2·93	3·98	3·20	3·33	3·46
% of plants with first two leaves opposite															
0	—	—	—	—	7·2	—	—	—	—	2·1	—	—	—	—	4·7
10	3·9	2·9	4·2	2·8	3·5	2·4	7·8	1·3	3·8	3·8	3·2	5·3	2·8	3·3	3·6
20	5·1	7·3	8·6	20·0	10·3	1·0	7·7	18·3	20·6	11·9	3·0	7·5	13·5	20·3	11·1
Mean	4·5	5·1	6·4	11·4	7·0	1·7	7·8	9·8	12·2	6·7	3·1	6·4	8·1	11·8	6·8
Days from emergence to date when third leaf reached length of 7 cm.															
0	—	—	—	—	43·51	—	—	—	—	45·73	—	—	—	—	44·62
10	46·34	44·97	46·94	44·90	45·79	44·11	44·36	44·44	44·11	44·26	45·23	44·67	45·69	44·51	45·02
20	45·04	43·81	43·51	45·44	44·45	42·58	44·90	42·99	44·11	43·65	43·81	44·36	43·25	44·78	44·05
Mean	45·69	44·39	45·23	45·17	44·45	43·35	44·63	43·72	44·11	44·31	44·52	44·51	44·47	44·64	44·55
Sum of squares of leaf lengths (cm.) on 3 Mar. (corrected for date of emergence)															
0	—	—	—	—	371	—	—	—	—	338	—	—	—	—	354
10	353	356	285	315	327	427	430	438	434	432	390	393	362	374	380
20	399	350	435	347	383	466	273	451	367	389	432	312	443	357	386
Mean	376	353	360	331	359	446	352	445	400	396	411	352	402	366	377
No. of leaves below first inflorescence															
0	—	—	—	—	11·10	—	—	—	—	11·13	—	—	—	—	11·11
10	11·10	11·05	11·15	11·30	11·15	11·10	11·20	11·00	11·15	11·11	11·10	11·12	11·08	11·23	11·13
20	10·95	11·25	11·35	11·30	11·21	11·05	11·10	11·45	11·30	11·22	11·00	11·17	11·40	11·30	11·22
Mean	11·03	11·15	11·25	11·30	11·16	11·07	11·15	11·23	11·22	11·16	11·05	11·15	11·24	11·26	11·16
Days from emergence to flowering															
0	—	—	—	—	88·4	—	—	—	—	88·4	—	—	—	—	88·4
10	87·6	87·2	89·3	87·7	87·9	87·9	88·4	84·7	86·6	86·9	87·7	87·8	87·0	87·1	87·4
20	86·1	87·5	86·3	88·4	87·1	86·0	86·5	86·6	88·5	86·9	86·1	87·0	86·5	88·4	87·0
Mean	86·8	87·4	87·8	88·0	87·7	87·0	87·4	85·7	87·5	87·2	86·9	87·4	86·7	87·8	87·4
Days from flowering to first gathering of ripe fruit															
0	—	—	—	—	72·2	—	—	—	—	71·6	—	—	—	—	71·9
10	72·1	70·3	72·8	71·7	71·7	70·2	71·3	74·0	73·6	72·3	71·1	70·8	73·4	72·7	72·0
20	71·4	71·8	76·0	70·9	72·5	69·8	70·9	73·7	73·2	71·9	70·6	71·4	74·8	72·0	72·2
Mean	71·8	71·0	74·4	71·3	72·1	70·0	71·1	73·8	73·4	72·0	70·9	71·1	74·1	72·4	72·1
Wt. of fruit per plant ripened before 20 June (g.)															
0	—	—	—	—	204	—	—	—	—	202	—	—	—	—	203
10	260	310	213	285	267	218	185	312	320	259	239	247	263	302	263
20	345	270	265	290	293	310	275	420	268	318	327	273	342	279	305
Mean	303	290	239	287	255	264	230	366	294	260	283	260	303	291	257
No. of fruits per plant ripened before 20 June															
0	—	—	—	—	3·0	—	—	—	—	2·8	—	—	—	—	2·9
10	4·0	4·3	4·1	4·6	4·2	3·5	2·9	4·1	4·5	3·7	3·7	3·6	4·1	4·6	4·0
20	4·8	3·8	4·0	4·3	4·2	4·4	3·4	5·4	3·2	4·1	4·6	3·6	4·7	3·7	4·2
Mean	4·4	4·0	4·0	4·4	3·8	3·9	3·2	4·7	3·9	3·5	4·1	3·6	4·4	4·2	3·7
Mean size of fruits (g.) ripened before 20 June (corrected for number of plants in row)															
0	—	—	—	—	67	—	—	—	—	72	—	—	—	—	69
10	64	72	47	55	60	62	66	71	70	67	63	69	59	63	63
20	71	70	67	65	68	70	81	77	81	77	70	76	72	73	73
Mean	67	71	57	60	65	66	73	74	75	72	66	72	66	68	68
Total wt. (kg.) of fruit per plant (corrected for number of plants in row)															
0	—	—	—	—	3·90	—	—	—	—	3·66	—	—	—	—	3·78
10	3·73	4·39	3·57	4·33	3·99	3·29	3·53	3·84	4·04	3·66	3·48	3·93	3·71	4·19	3·83
20	3·94	4·09	3·95	4·23	4·05	3·92	4·03	4·66	4·27	4·22	3·94	4·06	4·31	4·25	4·14
Mean	3·84	4·20	3·76	4·28	3·99	3·57	3·78	4·25	4·16	3·85	3·71	4·00	4·01	4·22	3·92
Total no. of fruits per plant (corrected for number of plants in row)															
0	—	—	—	—	59	—	—	—	—	56	—	—	—	—	58
10	59	66	56	70	63	56	51	55	62	56	57	58	56	66	59
20	59	60	60	60	60	63	59	63	65	63	61	60	61	62	61
Mean	59	63	58	65	61	59	55	59	64	58	59	59	59	64	60
Mean size (g.) of all fruits (corrected for number of plants in row)															
0	—	—	—	—	65	—	—	—	—	65	—	—	—	—	65
10	63	66	65	62	64	58	68	70	65	65	60	67	68	63	65
20	67	68	66	71	68	63	68	74	64	67	65	68	70	67	67
Mean	65	67	65	66	66	60	68	72	64	66	63	67	69	65	66

subjected to high temperatures (60–70° C.) for some hours, many of the plants may depart from the normal phyllotaxis in that the first two leaves (sometimes the next pair also) are almost or quite opposite. It was also found that this abnormality develops more slowly if the seeds are stored in a refrigerator.

It was thought that it might be of interest to discover whether chilling the imbibed seeds also affected the phyllotaxis. It was found (Table 1) that the proportion of plants with the first pair of leaves opposite among those grown from seeds chilled for 20 days was substantially greater than after 10 days' treatment or none. Though the effect of temperature alone did not reach the level of significance, the interaction of temperature with time of treatment did. The proportion of opposite-leaved plants was greater after treatment for 20 days at the higher than at the lower temperatures, but no such difference was to be found after only 10 days' treatment. The moisture conditions during germination had no effect. The analysis of variance is given in Table 2. No opposite-leaved seedlings were planted.

TABLE 2. *Analysis of variance: proportion of opposite-leaved plants* ($\sin^{-1}\theta$ *transformation*)

	n	Sum of squares	Mean square	*F*	*P*
Chilling time	2	513·8035	256·9018	6·19	<0·01
Chilling temperature	3	278·1313	92·7104	2·23	>0·05
Interaction: time × temperature	3	420·8172	140·2903	3·38	0·01–0·05
All other treatment effects	9	457·2363	50·8040	1·22	>0·05
Error (between seed boxes)	22	912·8775	41·4944	—	—
Total	39	2582·8658	—	—	—

(*c*) *Growth rate.* The interval from emergence to a definite stage in leaf development, namely, when the third leaf reached a length of 7 cm. from axil to apex, was taken as a measure of growth rate. The date at which this stage was reached was found by interpolation in the curve plotted from the weekly measurements of the leaf. The mean values for the various treatments are given in Table 1, and the points of interest from the analysis of variance in Table 3. The regression of this variable on the date of emergence was not significant.

TABLE 3. *Analysis of variance: days from emergence till leaf* 3 *reaches length of* 7 *cm.*

	n	Sum of squares	Mean square	*F*	*P*
Chilling time	2	7·6324	3·8162	1·55	>0·05
Interaction: chilling time × germination moisture	2	19·3750	9·6875	3·94	0·01–0·05
All other treatment effects	13	20·7291	1·5945	—	—
Error (between seed boxes)	22	54·1446	2·4611	—	—
Total	39	101·8811	—	—	—

Though the effects of time of chilling and moisture conditions during germination alone are not significant, their interaction is. That is, chilling increases the growth if the seeds are subsequently given adequate moisture supply during germination, but with a poor moisture supply decreases it. This may be because partial drying causes a set-back in the development of the chilled seeds which is not suffered by the less advanced control seeds.

(*d*) *Leaf area.* The leaf area was not measured directly. The lengths of five leaves were, however, measured regularly, and it was assumed that the sum of their squares would be fairly closely related to the leaf area of the plant. The sums of squares of the leaf lengths on 3 Mar. were calculated, i.e. for a date some 2 weeks later than the datum point for the growth-rate determinations. Since the leaf area increases approximately exponentially during the early development a logarithmic transformation was used in the analysis. The regression on emergence date was non-significant. The logarithmic means for the various treatments are given in Table 1, and the analysis of variance in Table 4.

TABLE 4. *Analysis of variance: sums of squares of leaf lengths (logarithmic transformation)*

	n	Sum of squares	Mean square	*F*	*P*
Chilling time	2	0·136207	0·068104	1·07	>0·05
Chilling temperature	3	0·641697	0·213899	3·37	0·01–0·05
Moisture during germination	1	0·419021	0·419021	6·61	0·01–0·05
Interactions:					
Time × temperature	3	1·116972	0·372324	5·87	<0·01
Time × moisture	2	1·166473	0·583237	9·19	<0·01
Temperature × moisture	3	0·388390	0·129463	2·04	>0·05
Temperature × time × moisture	3	0·230034	0·076678	1·21	>0·05
Error (between seed boxes)	22	1·401488	0·063431	—	—
Total	39	5·500282	—	—	—

The leaf area on the date in question was greater in the 'moist' series than in the 'dry' one; this difference is most marked in those chilled for 10 days. The effect of temperature of chilling is significant and also its interaction with chilling time. This is rather difficult to interpret, for in the 20-day series, but not in the 10-day series, the leaf area after treatment at 0 and 7° C. was much greater than at 2–3 and 8–11° C.

(*e*) *Position of first inflorescence.* The tomato plant grows sympodially, each inflorescence being terminal, and the axis being continued by the development of an axillary bud immediately below it. Goodall (1938) showed that the number of leaves whose initials are laid down before the initial of the first inflorescence can be affected by many external conditions, including seed treatments. Those seed treatments mentioned above as increasing the proportion of opposite-leaved plants also increase, in the normal plants, the number of leaves below the first inflorescence. As it is known (Purvis, 1934) that vernalization of winter cereals reduces the number of leaves formed before the ear, it was of interest to find whether chilling of tomato seeds might have a similar effect. The observations on this point are recorded in Table 1.

The inflorescences were formed at a slightly later morphological stage after the seeds had been subjected for 20 days to the higher chilling temperatures, but these differences do not reach significance (for temperature, and time × temperature interaction, *F* values are 2·69 and 2·25 respectively; significant level = 3·05). It is interesting to note, however, that these differences are in the same direction as might be expected from the position of the first leaves.

(*f*) *Date of flowering.* The mean intervals between the date of emergence and the opening of the first flower are tabulated in Table 1: the various treatments had no significant effect on this character.

Since each seed box sown from a given treatment was represented as nearly as possible equally in each row when the plants were planted in the greenhouse, any seed-box effect was confounded with the treatment effects in those observations (i.e. those on the cropping behaviour during most of the summer) made only row by row. It was therefore important to evaluate any seed-box effect during this period.

The variances within seed boxes and between seed boxes within treatments were calculated for four variables. For the sum of squares of leaf lengths, the seed-box effect was highly significant ($F = 6{\cdot}86$, significant level $= 1{\cdot}61$), but the seed-box conditions did not prove to influence the date of flowering ($F = 1{\cdot}31$, significant level $= 1{\cdot}67$). This was also true of the interval between flowering and fruiting ($F = 1{\cdot}22$, significant level $= 1{\cdot}59$) and of the weight of fruit gathered per plant before 20 June; here the variance within seed boxes was greater than that between seed boxes. Moreover, no effect of emergence date on any of the aspects of subsequent behaviour studied was found. Hence it was assumed that the seed-box conditions had ceased to affect the plants by the time they were planted in the greenhouse.

The unit of analysis for the variables so far discussed was the seed box. This could not be continued, however, after the plants had been for some time in the greenhouse—even for those variables for which the data for individual plants were available—since on account of the unfortunate equalization arrangement adopted in planting there was a risk that any block effect would be confounded with the treatment effects. This was avoided by analysing the results for the variables discussed below with the row as the unit, since the seed-box effect had been shown to be negligible.

TABLE 5. *Analysis of variance: weight of fruit per plant before 20 June*

	n	Sum of squares	Mean square	*F*	*P*
Block effect	3	862,792	287,597	22·00	< 0·01
Difference between chilled and unchilled series	1	139,752	139,752	10·70	< 0·01
Difference between 10-day and 20-day series	1	29,213	29,213	2·24	> 0·05
All other treatment effects	15	156,982	10,465	—	—
Error	68	887,446	13,051	—	—
Total	88	2,076,185	—	—	—

In respect of fruiting, the possibility had to be borne in mind that the reduction by mosaic disease in numbers of plants in many of the rows might affect the cropping of the remaining plants: accordingly, the regressions of the variables on the number of plants remaining in the row were tested. The values for the seven rows which had been completely destroyed were calculated by a missing plot method involving the minimization of error.

(*g*) *Date of first fruiting.* In the seventh section of Table 1 are given the mean periods in days from the opening of the first flower to the gathering of the first ripe fruit. None of the differences is significant.

(*h*) *The early crop.* In view of the economic importance of the yield during the first few weeks of cropping, the weights of fruit gathered between 2 and 19 June inclusive are treated separately. The mean weights of fruit per plant are given in Table 1. The chilling treatments have very considerably increased the crop during this period—the 10 days' treatment by 30 % and the 20 days' treatment by 50 %. The effect of the chilling is highly significant

(see Table 5), but none of the chilling treatments differ significantly among themselves, and the moisture conditions during germination have had no effect. The regression on the number of plants in the row was non-significant.

The next question to be answered was whether this increase in the early crop resulting from the chilling treatments was due to an increase in the number of fruits or in their mean size. The means of both these characters are given in Table 1, in the case of size corrected for number of plants in the row, the negative regression being highly significant. The analyses of variance are given in Tables 6 and 7. The number of fruits per plant during these weeks of June was highly significantly greater in the chilled than in the unchilled series; other differences in this character are non-significant. The fruits of the 10-day series are, however, significantly smaller than those of the unchilled series, and those of the 20-day series are significantly greater than either. The fruits in the 'moist' series are substantially larger than in the 'dry' series, this being marked only after the higher temperatures of chilling, when the seedling had reached a rather later stage in the incubator.

TABLE 6. *Analysis of variance: number of fruits per plant before 20 June*

	n	Sum of squares	Mean square	*F*	*P*
Block effect	3	162·5413	54·1803	28·75	<0·01
Difference between chilled and unchilled series	1	27·9549	27·9549	14·80	<0·01
Difference between 10-day and 20-day series	1	1·8320	1·8320	—	—
All other treatment effects	15	23·4994	1·5666	—	—
Error	68	128·4387	1·8888	—	—
Total	88	344·2663	—	—	—

TABLE 7. *Analysis of covariance: mean size of fruits before 20 June (and number of plants in row)*

	n	Sum of squares	Mean square	*F*	*P*
Block effect	3	84·299	28·100	—	—
Difference between 0-day and 10-day series	1	577·203	577·203	5·04	0·01–0·05
Difference between 20-day and other series	1	784·787	784·787	6·85	0·01–0·05
Germination moisture	1	1353·001	1353·001	11·81	<0·01
Interaction: chilling temperature × germination moisture	3	933·367	311·122	2·82	0·01–0·05
All other treatment effects	11	962·271	87·479	—	—
Regression	1	924·918	924·918	8·07	<0·01
Error	64	7333·967	114·593	—	—

Thus, the increase in yield of early fruit after chilling treatment of the seed is in the main to be ascribed to the increase in the number of fruits ripened during the early weeks of cropping, though the treatments also affect the fruit size.

(*i*) *The total crop.* In Table 1 there are also set out the mean weights per plant, the mean numbers per plant, and the mean sizes of the fruits ripened during the whole season. In all three cases, the negative regression on number of plants in the row was significant; the analyses of covariance for the former two variables are given in Tables 8 and 9. In respect of fruit size, no treatment effects were significant. The two significant effects in Tables 8 and 9 cannot be regarded as very definite, since both only just reach the level of significance.

The 20-day treatment has given the greatest weight of fruit per plant; the fruits of this series are also both more numerous and larger, though not significantly so. In the 'dry' series, the number of fruits is greatest after the 10-day treatment; in the 'moist' series, after 20 days.

TABLE 8. *Analysis of covariance: total weight of fruit per plant (and number of plants in row)*

	n	Corrected sum of squares	Mean square	*F*	*P*
Block effect	3	6·3344	2·1115	5·54	<0·01
Chilling time	2	2·4108	1·2054	3·16	0·01–0·05
Other treatment effects	15	6·3281	0·4219	1·11	>0·05
Regression	1	11·4916	11·4916	30·15	<0·01
Error	67	25·5402	0·3812	—	—

TABLE 9. *Analysis of covariance: total number of fruits per plant (and number of plants in row)*

	n	Corrected sum of squares	Mean square	*F*	*P*
Block effect	3	353·0573	117·6858	2·18	>0·05
Chilling time	2	193·7928	96·8964	1·80	>0·05
Interaction: chilling time × germination moisture	2	349·2124	174·6062	3·24	0·01–0·05
Other treatment effects	13	779·5278	59·9637	1·11	>0·05
Regression	1	650·0492	650·0492	12·05	<0·01
Error	67	3612·5304	53·9184	—	—

DISCUSSION

The results cited indicate that chilling imbibed seeds (var. Potentate) before germination at various temperatures between 0 and 11° C. has certain effects upon subsequent growth. The proportion of plants in which the first two leaves are opposite is increased; also the yield of fruit, particularly during the first few weeks of cropping, is increased. It is difficult to compare these results with those of earlier workers, as they used other varieties and some claimed to have found considerable varietal differences in response. Moreover, most of the temperature treatments they tried were outside the range of those used in the present investigation, and were applied after rather than before germination. The increases in yield found, usually considerably more than that in the total crop in the present work, appear mainly to have been ascribed to an advance in the dates of first flowering and fruiting, whereas in the Potentate plants described here the difference in date of flowering in the chilled and unchilled series is only about one day and is not significant.

The increase in yield found is sufficient to be of considerable economic importance, especially since it is concentrated at the beginning of the cropping season. The method adopted in chilling and germinating the seeds in Petri dishes and subsequently planting out the seedlings individually is hardly practicable on a commercial scale; but it is quite possible that the chilling process can be carried out after the seeds have been sown in seed boxes.

Chilling of seeds for 20 days appears to give definitely more favourable results than treatment for 10 days. Within the range of temperatures used there seems little to choose, though the higher temperatures (7–11° C.) gave rather better yields.

Summary

Tomato seeds (var. Potentate) were chilled in an imbibed state, but before germination, at temperatures of 0, 2–3, 7 and 8–11° C. for periods of 10 and 20 days; subsequently they were germinated, together with control seeds, in conditions both of restricted and of fully adequate moisture.

The plants grown from chilled seeds produced more fruit, particularly during the first few weeks of cropping, than those from unchilled seeds. Also their first two leaves were opposite in a greater proportion of the instances; this effect was more marked after treatment at the higher temperatures. Both leaf area at a given date and fruit size during the first weeks of cropping were greater in the series germinated with a full moisture supply than in those with restricted moisture. The dates of first flowering and fruiting were not affected by the treatments. The temperature differences appear to have had little effect.

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STUDIES IN BACTERIOSIS. XXV

STUDIES ON A BACTERIUM ASSOCIATED WITH LEAFY-GALLS, FASCIATIONS AND 'CAULIFLOWER' DISEASE OF VARIOUS PLANTS. PART IV. THE INOCULATION OF STRAWBERRY PLANTS WITH *BACTERIUM FASCIANS* (TILFORD)

By MARGARET S. LACEY

Bacteriological Laboratory, Imperial College of Science and Technology, London

(With Plate 1)

In Part I of this series Lacey (1936) gave an account of the isolation of a bacterium (later identified as *Bact. fascians* (Tilford)) from fasciated sweet peas, leafy galls on chrysanthemums, carnations and *Schizanthus retusus* and from strawberry plants affected by 'cauliflower' disease. All five strains of the bacterium produced typical fasciation of sweet-pea seedlings. During further work (Lacey, 1939) *Bact. fascians* was isolated from abnormal growths on a number of other plant species, and various strains of the bacterium were successfully inoculated on to chrysanthemums, carnations, schizanthus and other host plants. The strawberry strains of *Bact. fascians* were virulent to sweet pea, chrysanthemum, schizanthus, *Nicotiana glutinosa* and *N. tabacum*, on which they caused the production of galls identical with those induced by the inoculation of the specific host strain.

The inoculation of strawberry plants with *Bact. fascians*

The inoculation of strawberry plants, whether these inoculations were made with the strawberry strains or with other strains of *Bact. fascians* (chrysanthemum, sweet pea or carnation) did not give the high percentage of positive results obtained by the inoculation of other host plants with this bacterium. In these experiments runners, in most cases from 'Royal Sovereign' stock, were planted in pots and, after rooting, were inoculated either by pricking a needle smeared with culture into the crown or by thoroughly wetting the crown with a water suspension of the organism or by both methods combined. Control plants were pricked with a sterile needle or treated with sterile water. Inoculations were made at intervals throughout the year so that plants in all stages of growth were treated during the course of the experiments. In brief, of 110 plants inoculated during a period of 18 months, twelve died from unknown causes soon after inoculation, twelve (var. Sir Joseph Paxton) were attacked by root-rot and discarded (the controls being equally affected) and twenty-two of the remaining eighty-six plants developed 'cauliflower' symptoms to a greater or less degree at varying periods after inoculation, the controls being free from disease. Pl. 1, fig. 1, shows the type of abnormal growth produced. In this case the infection developed slowly, the photograph being taken a year after inoculation when normal growth had ceased. To the left of the crown is a typical malformed leaf, consisting of three tiny leaflets attached to a short, swollen petiole. In the centre a sessile flower structure has been produced; this has no petals or carpels and the stamens are scattered among the sepals. The remaining growth consists of undifferentiated gall tissue. This plant survived a further six months, during which time one or two more rudimentary leaves were produced. Similar results

were obtained in a few other cases, death of the whole plant ensuing 18 months to 2 years after inoculation. (*Bact. fascians* was isolated in large numbers from the crown of a plant which died 2 years after inoculation.) In other cases the primary crown died after producing hypertrophied growth, but healthy lateral crowns developed. The majority of the plants, however, after producing a few deformed leaves and fasciated side-shoots, recovered and showed no further signs of disease during the time ($1\frac{1}{2}$–$2\frac{1}{2}$ years) they were kept under observation. The recovery of strawberry plants slightly affected by 'cauliflower' disease and their subsequent healthy growth frequently occurs in the field under natural conditions, but the irregularity and variability of the amount and degree of infection obtained in these inoculation experiments make the results inconclusive. For example, of twelve plants inoculated in April 1937, six developed signs of 'cauliflower' disease 2–5 months later. The following April two of these were seriously diseased, the leaves being reduced to short, thick, tapering petioles, red in colour, with absent or rudimentary leaflets; these plants died a few months later. The primary crowns of three of the other diseased plants died, but weak lateral crowns developed, and the sixth plant made complete recovery. As these April inoculations were more successful than those made the previous summer and autumn, spring appeared to be the time at which infection most readily occurred. But when, in April 1938, ten more strawberry plants were inoculated, a negative result was obtained, none showing any signs of disease during the 18 months they were kept under observation.

As the experiments with plants grown from runners gave such irregular results further trials were made using young plants grown from seed of vars. 'Royal Sovereign', 'Large English', and 'St Jean'. The 'Royal Sovereign' seed germinated well but most of the young seedlings died. In one test, however, four plants survived and developed normally. Three of these were inoculated with *Bact. fascians* in May 1939, when about 10 months old, the fourth being kept as control. One month later, two of the inoculated plants had growths of abnormal leaves, consisting of much reduced, malformed leaflets on short, stout petioles, and after 3 months all three inoculated plants showed marked signs of 'cauliflower' disease (Pl. 1, fig. 2). The control plant was normal. In June 1939 three more young 'Royal Sovereign' plants were inoculated, and one of these showed 'cauliflower' symptoms 6 weeks later. No further records of these plants were obtained.

The 'Large English' strawberry seeds grew well but the seedlings were found to be but slightly susceptible to *Bact. fascians* infection. As the result of the inoculation of plants of this variety small fasciated side shoots occasionally developed but the main growth was not affected.

The 'St Jean' variety, however, not only grew easily from seed, but was susceptible to infection with *Bact. fascians*. This variety was therefore used in 1938 and 1939 for inoculation experiments, details of which are given below.

Inoculation of 'St Jean' strawberry seedlings

(1) 1938 *inoculations*. At intervals from May to August 1938, thirty-six 'St Jean' strawberry seedlings were inoculated with cultures of *Bact. fascians* by means of needle pricks into the crown but seven of these died soon after inoculation, possibly as the result of the wounding. Of the remaining twenty-nine, twenty exhibited signs of 'cauliflower' disease shortly after inoculation, fasciated growths being apparent 3–8 weeks later. Three plants

were so severely affected that death ensued 5–10 months after inoculation and six were badly diseased but still living after 14 months. The remaining eleven were only slightly affected; four of these recovered and developed normal growth during the summer of 1939, while seven remained small and unhealthy looking but showed no definite 'cauliflower' symptoms a year after inoculation. None of the control plants, which were pricked in the crown with a sterile needle, developed fasciated growth at any time during the 18 months they were kept under observation. One control plant, however, was a poor, unhealthy specimen, with curled, mottled leaves. The appearance of this plant suggested virus disease.

The histories of the three most severely diseased plants are summarized below:

Plants 1 *and* 2:

11. vii. 38.	Inoculated by needle-pricks (1) with a strawberry strain, (2) with a sweet pea strain of *Bact. fascians*.
8. viii. 38.	Gall-like growths surround the stems at soil level.
30. viii. 38.	Considerable gall tissue at soil level; plants ceased normal growth.
26. ix. 38.	Large 'cauliflower' growths at the side of the primary crown (Pl. 1, fig. 3).
10. x. 38.	Galls rotting, no further growth.
1. ii. 39.	Plants dead.

Plant 3:

4. v. 38.	Inoculated by needle-prick with a strawberry strain of *Bact. fascians*.
29. vi. 38.	Two minute leaves with short, stout, bright red, tapering petioles and rudimentary lamina.
19. vii. 38.	New leaves have long, thin, red, hairless petioles and small, deformed lamina.
30. viii. 38.	Three weak crowns bearing fasciated leaves.
24. x. 38.	Abnormal growth continued; numerous small leaves with greatly reduced lamina and long, thin, hairless petioles.
18. i. 39.	Photograph taken (Pl. 1, fig. 4). Control plant A, inoculated plant B. Compare absence of hairs on the petioles of the inoculated plant with the mat of hairs covering petioles of the control plant.
18. iv. 39.	Plant dead.

The effect of the inoculation of *Bact. fascians* on plant 3 was different from that produced on the first two plants. In the latter, gall tissue was produced at the side of the primary shoot, which soon ceased growth; in plant 3 there was no definite gall production but abnormal growth of the primary shoot, the character of which resembled the disease known as 'red-plant'. (*Bact. fascians* has been isolated in large numbers from a natural infection of 'red-plant' of strawberry.)

Pl. 1, fig. 5 shows the development of small, almost sessile leaves in the centre of the crown of a fourth plant of this series, 1 year after inoculation. A control plant of the same age is shown in Pl. 1, fig. 6. Another inoculated plant, which was only slightly affected the first season, never produced any definite 'cauliflower' symptoms, but a year after inoculation the plant was dwarfed, with small, bluish green, somewhat 'leathery' leaflets and very short petioles.

(2) 1939 *inoculations*. In the spring of 1939 fresh isolations of *Bact. fascians* were made from naturally infected strawberry plants (var. Oberschlesien) which were very severely affected by 'cauliflower' disease. Four of the newly isolated cultures (A, B, D and E) were used for the inoculation of thirty-six 'St Jean' strawberry seedlings in June 1939, nine plants being inoculated with each culture by gently pricking a fine needle smeared with the

organisms into the tip of the crown: eighteen control plants were similarly pricked with a sterile needle. The results are summarized below:

5. vi. 39.	Plants inoculated.
13. vi. 39.	A 1 has fasciated growth in the axil of the oldest leaf.
23. vi. 39.	A 2: Three new leaves have small, deformed laminae.
26. vi. 39.	A 1, 3, 4 and 5; D 1, 2 and 3; E 1, 2 and 3 have fasciated axillary growth. The 9 B and 18 control plants all normal.
3. vii. 39.	A 5; B 1; D 4 and 5; E 4 have definite gall formation at the side of the crown (Pl. 1, fig. 7).
10. vii. 39.	A 2, 3, 6, 7 and 8 have malformed leaves; each new leaf smaller than the preceding one.
17. vii. 39.	A 2; B 1 and 2; D 2 and 3; E 2: Gall tissue developed at soil level (Pl. 1, fig. 8).
24. vii. 39.	E 5 positive.
10. viii. 39.	A 9; B 3 and 4; D 1, 5 and 6; E 5: 'Cauliflower' growths at soil level.
	A 6 and 8: Primary shoot divided into four weak crowns bearing leaves with long, thin, hairless petioles and reduced laminae.
	A 3, 5 and 7: New leaves very small.
	B 5: Very tiny, flat growth.
	E 6: Small leaves with red veins.
	18 controls: No abnormal growth; 16 strong, healthy plants, 2 small, attacked by mildew.

In all, thirty of the thirty-six inoculated plants developed some type of abnormal growth 1–10 weeks after inoculation (all 9 A's, 6 B's, 7 D's and 8 E's). Of these thirty, fifteen developed definite gall tissue and fifteen showed abnormalities such as the division of the primary shoot into several poor, weak secondary crowns and the production of small, fasciated leaves.

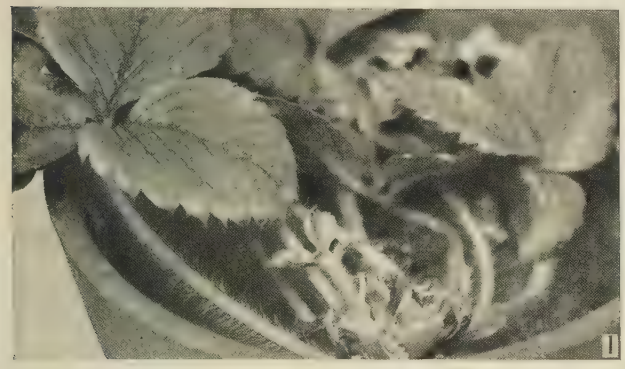
Experiment to determine if *Bact. fascians* can infect strawberry seedlings in the absence of wounds

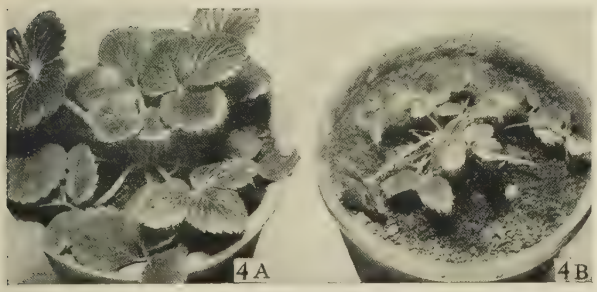
In the previous experiments, *Bact. fascians* was introduced into the strawberry crowns by needle pricks. To determine if the organism can cause disease in the absence of wounds, twenty-four 'St Jean' strawberry seedlings were infected by brushing a camel-hair brush dipped in a water suspension of *Bact. fascians* over the shoots: twenty-four control seedlings were similarly treated with sterile water. Five weeks later, two of the infected seedlings had a mass of gall tissue round the stems at soil level; two more were positive after 10 weeks and another three after 12 weeks. The remaining infected plants, and all the controls, appeared free from disease when the last observation was made, 4 months after inoculation. The number of positive infections, seven out of twenty-four, though considerably less than that obtained by wounding, shows that, as had already been found to be the case in the fasciation of sweet peas, leafy gall of *Nicotiana*, etc., *Bact. fascians* can produce disease in young strawberry plants in the absence of wounds.

Further observation of these plants and of the other seedling inoculations was prevented by the outbreak of war, but the results obtained afford definite evidence that *Bact. fascians* can be the causal agent in the production of abnormal growths on young strawberry plants.

Summary

Attempts to produce 'cauliflower' disease of strawberry by the inoculation of *Bact. fascians* into plants grown from runners gave inconclusive results: 25 % of the inoculated plants developed some signs of 'cauliflower' disease at various times, but although a few





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were so severely affected that death ensued the majority recovered and subsequently showed no signs of abnormal growth. The inoculation of seedling strawberry plants with *Bact. fascians* gave definite positive results, abnormal growths being produced in 76 % of the infected plants. *Bact. fascians* can cause gall production on young strawberry plants in the absence of wounds.

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EXPLANATION OF PLATE 1

Fig. 1. Strawberry plant 1 year after inoculation with *Bact. fascians*. × ⅓.
Fig. 2. 'Royal Sovereign' strawberry seedling, 3 months after inoculation with *Bact. fascians*. × ½.
Fig. 3. 'St Jean' strawberry seedling 10 weeks after inoculation with *Bact. fascians*. × 1.
Fig. 4. 'St Jean' strawberry seedlings. A, control plant; B, 6 months after inoculation with *Bact. fascians*. × ½.
Fig. 5. 'St Jean' strawberry seedling, 1 year after inoculation with *Bact. fascians*. × 1.
Fig. 6. Control plant of same age as fig. 5. × ½.
Fig. 7. 'St Jean' strawberry seedling, 5 weeks after inoculation with *Bact. fascians*. × 1.
Fig. 8. 'St Jean' strawberry seedling 8 weeks after inoculation with *Bact. fascians*. × 1.

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PRESENCE OF VIRUS IN THE PRIMORDIAL MERISTEM

By F. M. L. SHEFFIELD, D.Sc.
Rothamsted Experimental Station, Harpenden, Herts

Viruses have been shown to be present and intracellular inclusions have been found in many tissues of infected plants. No one has attempted to recover virus from the meristematic layers of the growing point and only on one occasion have intracellular inclusions been recorded there (Goldstein, 1926). Goldstein regularly found them in the leaf primordia of tobacco with mosaic whilst the cells were still in active division. That virus is present in some cells immediately after division ceases is suggested by the inhibition of the development of the proplastids in aucuba mosaic infected plants (Sheffield, 1933). I have examined living and fixed preparations of the growing points of tobacco, tomato and *Solanum nodiflorum* infected with tobacco and aucuba mosaic viruses, tobacco with *Hyoscyamus* virus 3 and severe etch virus, and dahlia with mosaic virus, but have never seen any abnormalities. Similarly Clinch (1932) was unable to find any inclusions in the meristem of virus-infected potatoes although they were present in the differentiated tissues. I found that the intranuclear inclusions of severe etch virus appeared in the young leaves earlier than did the cytoplasmic inclusions produced by any of the viruses mentioned but not until all *normal* nuclear division had ceased (Sheffield, 1941). It seemed possible that the absence of inclusions from the primordial meristem might be due to failure of the virus to penetrate there, and experiments were made to test this.

Meristematic tissue was dissected out from the apices of plants infected with either tobacco or aucuba mosaic virus, care being taken to eliminate all cells in which active nuclear division had ceased: with roots, the root cap was removed. The roots and shoots selected were of such a size and shape that the dissected growing points could be regarded as hemispheres of about 100μ diameter (or $\frac{1}{4} \times 10^{-6}$ c.c. in volume). Each dissected growing point was washed several times by flooding with water, then removed to a sterile slide where it was cut into fragments before suspension in a measured volume of water. The material was stored at 4° C. until sufficient was collected, when the resulting suspension was inoculated to *Nicotiana glutinosa* leaves. Comparison was made with an equal quantity of a comparable dilution of crude sap from an old leaf or from roots of the plant from which the growing points were obtained. A water control also was used. Further details of the experiments are given together with the results in Table 1.

Table 1. *Infectivity of apical meristems from virus-infected plants*

Host	Virus strain	Origin of growing points	20 growing points in 0·5 c.c. water	Crude sap 1 in 10^5	Water
Tomato	Tobacco mosaic	Shoot	3	6 (leaf)	0
Tomato	Tobacco mosaic	Shoot	8	29 (leaf)	0
Tobacco	Tobacco mosaic	Root	7	24 (leaf) 14 (root)	0
Tomato	Tobacco mosaic	Shoot	13	16 (leaf) 12 (root)	0
Tomato	Aucuba	Shoot	31	28 (leaf)	0
		Root	23	17 (root)	

(Figures show numbers of lesions on *Nicotiana glutinosa*)

The results show that virus was undoubtedly present in the primordial meristem of both roots and shoots of plants infected with ordinary tobacco mosaic virus or with the aucuba strain. The absence of intracellular inclusions must therefore be attributed to other causes.

Similar tests were also made with apical meristems from roots and shoots of tomatoes infected with severe etch virus. Thirty tobaccos inoculated with suspensions all remained healthy. The virus content of sap from plants infected with severe etch virus is however, only about one-thousandth of that from tobacco plants with mosaic. The fact that the inoculum was too dilute probably explains the result rather than that the tissues were virus free.

Summary

It was thought that the absence of intracellular inclusions from the apical meristem of infected plants might be due to failure of the virus to penetrate there. However, if this tissue is dissected out from shoots or roots of plants with tobacco or aucuba mosaic infections can be obtained from it.

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SELECTION OF BACTERIAL FOOD BY SOIL FLAGELLATES AND AMOEBAE

By B. N. SINGH

Department of Soil Microbiology, Rothamsted Experimental Station, Harpenden, Herts

Singh (1941*a*) showed that soil amoebae can select food among different kinds of bacteria: some bacteria are preferred, and others are non-edible even if they are the only food available. No definite relationship between edibility and the morphological or physiological characteristics of bacteria was found. *Serratia marcescens* produces an exo-toxin which prevents other nearby edible bacteria from being eaten by the amoebae.

Large numbers of flagellates are found in the trophic condition in suitable soils, but little is known of their selectivity of bacterial food, though many lead a holozoic existence. Experiments were made to see whether the preference for different kinds of bacteria by *Cercomonas crassicauda*, a common soil flagellate, resembles that of the soil amoebae. This comparison was made with the strains of bacteria, mostly from soil, previously tested on amoebae (Singh, 1941*a*), and also on nodule bacteria (*Rhizobium*) and on certain plant pathogens.

Material and methods

To test the selectivity in bacterial food by soil amoebae the method of radiating streaks described by Singh (1941*a*) and the same two species of amoebae were employed.

Cercomonas crassicauda was obtained from Barnfield farmyard-manured soil (Rothamsted Experimental Station, Harpenden). A little soil was diluted in sterile hay infusion and incubated at 20° C. for a few days till a large number of protozoa developed. A pure line culture was obtained by washing a single *Cercomonas* several times in sterile salt solution and placing it in the centre of a bacterial circle of a given species as food. The term 'bacterial circle' designates the growth obtained by spreading a suspension of the bacteria to be tested over a circular patch about 1 in. diam. on the surface of a poured plate of sterile agar medium. Micro-pipettes were used for isolating and transferring *Cercomonas* from one washing solution to the next. Throughout the experiment with *Cercomonas*, nutrient agar containing 0·75 % agar was used. It is important to reduce the percentage of agar used to cultivate flagellates on solid medium, because these need more moisture, in the solid medium, than amoebae: 1·5 or 2 % agar was not suitable to culture *Cercomonas*. The food preferences of *Cercomonas* were tested either by the method of radiating streaks (Singh, 1941*a*) or by adding the flagellate to the centres of prepared 'bacterial circles' growing on agar platings. In all the experiments young bacterial cultures of the same age were used and the temperature of incubation was 19–20° C.

Cercomonas was tested on the following strains of bacteria, mostly from soil: 07, 08, 1734, 1912, 2006, S21, N16(i), λT20, 2881, 0312, 0746, 4022, 4031, 4045, 5431, 5654 and R. The characters of these strains and the reactions of amoebae to them are given in a previous paper (Singh, 1941*a*). Both amoebae and *Cercomonas* were tested on the following plant pathogenic bacteria and strains of *Rhizobium*.

Plant pathogenic bacteria. Phytomonas tumefaciens (Smith & Townsend), 1997, 4752 and 5942, *P. hyacinthi* (Wakker) 5943 and 387, *P. mori* (Boyer & Lambert) 1989, *P. phaseoli* (Erw. Smith) 5241, *P. syringae* (Van Hall) 5944, *P. malvacearum* (Erw. Smith) 4756, *Erwinia tracheiphilum* (Erw. Smith) 3725, *E. carotovorum* (Jones) 5945, *E. solanisaprum* (Harrison) 385.

Rhizobium strains

Effective strains are those that fix adequate quantities of nitrogen for the use of the host legume: 'avirulent' strains have lost the power to infect the host plant through prolonged laboratory culture.

Clover	A1	Effective
	Coryn	Ineffective
	A11	Avirulent
	Bact. A	Avirulent
	2057	Effective
	20272	Ineffective
	202	Avirulent
	C3	Ineffective
Pea	317	Effective
	310	Ineffective
	P11	Effective
	P11R	Effective
Lupin	Wisconsin 3	
Lucerne	A and H	
Soy bean	505	Effective
	50	Ineffective

In addition, three other strains of a soil bacterium were tested in order to examine the effect of slimy capsules. Bacteria E and EW are closely similar soil bacteria that produce large slimy capsules. Bacterium E was described by Kleczkowska *et al.* (1940). EW was isolated from Woburn soil and is morphologically similar to E. B2 is a mutant of E and does not produce slimy capsules but is otherwise similar to the parent form both culturally and in serological behaviour.

Observations

Table 1 shows the comparative edibility of the various strains of bacteria tested to the three species of protozoa. Strains described as 'partly eaten' are eaten over a small area for a few days, after which most of the protozoa die or encyst.

The two species of amoebae resemble one another closely in their food preferences. No bacterial strain inedible by the large amoeba could be eaten by the small species, but two strains of *Rhizobium* inedible by the small amoeba were partially eaten by the large species, which slowly but completely consumed the strain of *Phytomonas tumefaciens*, 1997 that was inedible by the small amoeba.

With *Cercomonas* it was difficult to estimate how rapidly the test bacteria were consumed. This was due partly to the comparative lack of motility of the flagellate over a solid medium and partly to the necessity of using a weaker agar, over which motile bacteria often spread until they covered the whole agar surface. Owing to this difficulty, the class of test bacteria 'completely eaten' could not be subdivided into those readily and those slowly consumed.

The flagellate showed considerable resemblance to the amoebae in its food preference, but it could feed on some strains of bacteria not eaten by the amoebae, and it consumed the edible strains more slowly and usually less completely. The resemblances and differences in food preference between the large amoeba and *Cercomonas* are summarized below:

Bacterial strains partly or completely eaten by both ...	19
Strains eaten by neither	18
Strains eaten by the amoeba but not by *Cercomonas* ...	1
Strains eaten by *Cercomonas* but not by the amoeba ...	10
Total strains tested	48

There are thus thirty-seven strains about whose palatability both the protozoa agree and eleven strains, or 22·9 %, about which they differ.

TABLE 1. *Selectivity in bacterial food by amoebae and* Cercomonas

(N) = slightly eaten on rare occasions or not eaten; S = slowly eaten; R = readily eaten

Bacterial strain	Edibility by large amoeba			Edibility by small amoeba			Edibility by *Cercomonas*		
	Completely eaten	Partly eaten	Not eaten	Completely eaten	Partly eaten	Not eaten	Completely eaten	Partly eaten	Not eaten
Miscellaneous strains of bacteria, mostly from soil									
07	R	.	.	R	.	.	C	.	.
08	R	.	.	R	.	.	.	P	.
1734	R	.	.	R	.	.	C	.	.
1912	R	.	.	R	.	.	C	.	.
2006	R	.	.	R	.	.	C	.	.
S21	R	.	.	R	.	.	C	.	.
λT20	R	.	.	R	.	.	C	.	.
N16(i)	S	.	.	S	.	.	C	.	.
4045	S	.	.	S	.	.	C	.	.
0312	.	.	N	.	.	N	.	P	.
0746	.	.	N	.	.	N	.	.	N
4022	.	.	N	.	.	(N)	C	.	.
4031	.	.	N	.	.	(N)	.	P	.
2881	.	.	N	.	.	N	.	.	N
5431	.	.	N	.	.	N	.	.	N
5654	.	.	N	.	.	N	C	.	.
R	.	.	N	.	.	(N)	C	.	.
E	.	.	N	.	.	N	.	.	N
EW	.	.	N	.	.	N	.	.	N
B2	.	.	N	.	.	N	.	.	N
Strains of *Rhizobium*									
Clover A1	.	.	N	.	.	N	.	.	N
Clover Coryn	.	P	.	.	.	N	.	.	N
Clover A11	.	.	N	.	.	N	.	.	N
Clover Bact. A	.	.	N	.	.	N	.	P	.
Clover 2057	.	.	N	.	.	N	.	.	N
Clover 20272	.	.	N	.	.	N	.	.	N
Clover 202	.	.	N	.	.	N	.	P	.
Clover C3	.	.	N	.	.	N	.	.	N
Pea 317	.	.	N	.	.	N	.	.	N
Pea 310	.	.	N	.		N	.	.	N
Pea 11	.	.	N	.		N	.	.	N
Pea 11R	.	.	N	.		N	.	.	N
Lupin Wisconsin 3	.	.	N	.		N	.	.	N
Lucerne A and H	.	P	.	.		N	.	P	.
Soy bean 505	.	.	N	.		N	.	.	N
Soy bean 50	.	.	N	.		N	.	.	N
Plant pathogens									
4752	R	.	.	R	.	.	C	.	.
3725	R	.	.	R	.	.	C	.	.
5945	R	.	.	R	.	.	C	.	.
1989	R	.	.	R	.	.	C	.	.
5944	R	.	.	R	.	.	.	P	.
4756	R	.	.	R	.	.	C	.	.
1997	S	.	.	.	.	N	.	P	.
5942	S	.	.	R	.	.	.	P	.
385	S	.	.	S	.	.	C	.	.
5943	.	.	N	.	.	N	C	.	.
387	.	.	N	.	.	N	C	.	.
5241	.	.	N	.	.	N	.	P	.

The bacteria tested fall into three groups. One comprises a wide variety of organisms, mostly from soil, whose morphology and growth characters differ widely, and about half of which are eaten by the amoebae. The second group is composed of strains of *Rhizobium*, closely resembling one another and nearly all inedible. The third group consists of plant pathogens most of which can be eaten by the amoeba and all by the *Cercomonas* either partly or completely. It may thus appear at first sight that the strains tested do not supply a valid estimate of the agreement in food preference between the amoeba and the flagellate since more than half of these strains belong to two specialized groups. But within the first group of unrelated bacteria fifteen strains are either edible or inedible by both the amoeba and the *Cercomonas*, while five produce different reactions in the two protozoa, which thus differ in their preferences as regards 25 % of the strains in this group. Within the two specialized groups the two protozoa differ as regards 21·4 % of the strains. Differences in reaction between the amoeba and the flagellate are thus as likely to occur towards a strain belonging to a group of related and specialized organisms as towards one taken from a collection of unrelated bacteria.

Serratia marcescens, strain 2881, produces an exo-toxin affecting amoebae (Singh, 1941*a*). Exo-toxins, similarly capable of preventing amoebae from eating otherwise edible bacteria, were found to be produced by all the strains of plant pathogens that were inedible by amoebae. None of these exo-toxins could be shown to affect *Cercomonas*, which could eat all the strains of plant pathogens partly or completely.

There seemed to be no correlation between edibility and slime production by the bacteria. The *Rhizobium* strains produce slime in varying amounts and most are not eaten, but the soil bacteria E, EW and B2 are all inedible although the first two have slimy capsules, which are lacking in the mutant from B2. Also strain Lucerne A and H is slimy but is eaten both by the large amoeba and by *Cercomonas*. To test the possible effect of bacterial slime on the feeding of the protozoa, slime from a culture of Pea *Rhizobium*, strain 310, was separated by filtration and precipitation with alcohol. After drying, the slime was redissolved in sufficient water to make a thick paste which was mixed with a growth of edible bacteria growing on the surface of an agar plating. The bacteria thus treated were eaten both by the amoebae and by the *Cercomonas*. It is still possible, however, that slime naturally produced and in more intimate contact with the bacteria may account for the inedibility of the *Rhizobium* strains.

There seems to be some connexion between pigment formation and unpalatability. In the first group of unrelated bacteria, seven strains are inedible to amoebae and two are but slowly eaten out of a total of ten pigmented strains, while six of the seven unpigmented strains are readily eaten, the seventh and inedible strain being *Radiobacter*, which is closely related to *Rhizobium* whose unpalatability is due to a different cause.

The only strain outside the group of plant pathogens that produced exo-toxin in *Serratia marcescens* is strain 2881, which produces a diffusible pigment. There is thus a possibility that pigment formation by bacteria exerts a protective action against protozoal attack.

Discussion

Work on the selectivity among bacterial food by soil amoebae and a soil flagellate in 'pure-mixed' culture confirms the idea that the interrelationship of soil protozoa and bacteria is not a simple one. If amoebae and flagellates behave differently towards certain bacterial

food, the effects in soil of the phagocytic action brought about by these two important groups of soil protozoa may be different. It was shown by Singh (1941*a*) that amoebae can select their food in sterilized soil, but in the present state of our knowledge it is difficult to discuss this point. Towards certain groups of bacteria it seems that soil protozoa (amoebae, ciliates and flagellates) have little or no phagocytic action, as in the case of the different strains of nodule bacteria. Singh (1941*b*) also showed that a ciliate *Colpoda steinii* does not eat bacterium Pea 310.

Hino (1934) found that a large inoculum of the ciliate *Colpoda saprophila* may destroy *Bacillus aroideae* to such an extent that the plants are free from the attack of the pathogens. He carried out his experiments in liquid medium. If this be true it would be interesting to extend this knowledge to soil amoebae and flagellates which are known to lead an active and trophic existence in large numbers in suitable soils. The present work shows that certain plant pathogenic bacteria are eaten by flagellates and amoebae whilst others are not and it may thus be possible that the incidence of particular plant diseases caused by bacteria may be influenced by the presence of protozoa in the soil.

Summary

The food preferences of two soil amoebae and a soil flagellate, *Cercomonas crassicauda*, were compared as regards forty-eight strains of bacteria, which included a miscellaneous group mostly from soil, a group of *Rhizobium* strains and a group of plant pathogens. The amoebae were able to eat about half of the strains belonging to the miscellaneous group and most of the plant pathogens. Nearly all the strains of *Rhizobium* were inedible. The *Cercomonas* ate a rather larger number of strains than did the amoebae and differed from the large amoeba in its food preference as regards eleven out of the forty-eight bacterial strains. The plant pathogens that were inedible by amoebae produced an exo-toxin harmful to amoebae, but without apparent effect on *Cercomonas*, which could eat all these strains partly or completely. Slime produced by certain strains of the bacteria did not inhibit the feeding of the protozoa.

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THE SEASONAL INCIDENCE, OCCURRENCE AND DISTRIBUTION OF PROTOZOA IN THE BACTERIA BED PROCESS OF SEWAGE DISPOSAL

By A. NEVILLE BARKER, M.Sc.
Department of Zoology, The University, Leeds

(With 1 Text-figure)

Attention has recently been directed to the fauna of the sewage bacteria beds, particularly since their efficiency has been found to be related to the organisms present. The medium of the beds is clothed with an abundant protozoan fauna, and it is probable that this has an important relation to the water changes. It has, however, not been investigated in detail, Johnson (1914) merely listing the forms encountered in the beds at Wakefield and grouping them according to the degree of impurity they tolerate. In other purification processes more attention has been given to these organisms. Lackey (1924, 1925 *a*, *b*) recorded their presence in detail in the Imhoff tank process, while Lockett (1928) and Barritt (1940) studied them in activated sludge.

The present study is a preliminary survey to further investigations designed to assess the role of the Protozoa in the bacteria bed process and forms part of a general survey of the fauna of bacteria beds in progress at Leeds, Reynoldson (1939*a*) and Lloyd *et al.* (1940) having ascertained the influences of the Enchytraeidae and the Insecta respectively in the general process of purification.

Material and method

Observations were made at the Knostrop Sewage Works, Leeds, where the sewage is of medium strength, highly mixed in character and almost neutral in reaction. The system comprises screens, detritus tanks, sedimentation tanks, gravel bacteria beds (Lloyd *et al.* 1940), and humus settlement tanks. Both sedimentation and humus tanks are emptied and cleaned weekly.

Samples have been taken from the sedimentation tanks, bacteria beds and humus tanks throughout a complete year, and fluctuations in the forms present and their numbers have been determined to provide a complete record of the seasonal changes. For this purpose the following routine observations were made:

(1) In the sedimentation and humus tanks weekly liquid samples were taken at the surface at a definite time and place each week. Samples were also taken occasionally at depths of 1, 2, 4, 6 and 8 ft. by a special apparatus enabling uncontaminated samples to be obtained. In addition, slides were suspended in vertical frames and removed weekly for examination either directly or after fixation.

(2) In the bacteria bed a frame containing sixty slides was buried 10 in. in the medium and one slide removed at fortnightly intervals, whilst on alternate weeks suspensions were obtained by adding bed effluent to wide-necked jars of 70 ml. capacity filled with medium from the beds. After vigorous shaking the liquid was decanted into the sampling jar.

(3) Liquid samples were taken from the various channels of the system.

(4) Daily temperature records of each site were kept.

Before examination it was necessary to concentrate a sample to one-twentieth of its bulk by centrifuge. To estimate the number of organisms in the concentrate a drop was transferred to the chamber of a Thoma haemocytometer which was examined using an eyepiece masked to give a square field 1 mm. across with the $\frac{2}{3}$ in. objective and $\frac{1}{25}$ sq. mm. with the $\frac{1}{6}$ in. As the flagellates were most numerous 0·23 sq. cm. of the chamber was examined using the lower power, while for the ciliates and rhizopods 1·15 sq. cm. were examined. Minute flagellates were counted under the higher power by

the examination of eleven representative fields. A density of eight organisms per ml. and upward could be recorded with the low power lens and from 113 per ml. upwards with the high power. To examine the slide films, eleven representative fields were selected and viewed with the low power and high power; the number of organisms per sq. cm. was then calculated.

General observations

The genera and number of species of Protozoa found in the tanks and bacteria beds are summarized in Table 1. The density of population in the bacteria beds and humus tanks throughout the season is shown in Table 2. Distinct trends were noted, but these were lacking in the sedimentation tanks where the abundance varied sporadically.

Table 1. *Table of the genera of Protozoa represented in the sedimentation, bed and humus stages of the Knostrop Works*

SARCODINA	
Rhizopoda	
Amoeba (4)	S.B.H.
Vahlkampfia (2)	S.B.H.
Cochliopodium (1)	S.B.H.
Arcella (1)	B.H.
Difflugia (1)	B.H.
Euglypha (1)	B.H.
Trinema (1)	B.H.
Heliozoa	
Actinophrys (1)	H.
MASTIGOPHORA	
Pantostomina	
Mastigamoeba (2)	S.B.H.
Dimastigamoeba (1)	S.
Protomonadina	
Oikomonas (2)	S.B.H.
Monas (2)	S.B.H.
Pleuromonas (1)	S.B.H.
Bodo (2)	S.B.H.
Cercobodo (2)	S.B.H.
Trepomonas (1)	S.B.H.
Polymastigina	
Hexamitus (1)	S. H.
Euglenoidina	
Euglena (1)	S.B.H.
Astasia (1)	S.B.H.
Distigma (1)	H.
Menoidium (1)	B.H.
Peranema (1)	H.
Petalomonas (1)	S.B.H.
Anisonema (1)	B.H.
Phytomonadina	
Chlamydomonas (1)	H.
Polytoma (1)	S.B.H.
CILIOPHORA	
Holotricha	
Holophrya (1)	S.B.H.
Chaenia (1)	H.
Spathidium (1)	H.
Prorodon (1)	B.H.
Amphileptus (2)	B.H.
Lionotus (2)	B.H.
Chilodon (2)	S.B.H.
Colpidium (1)	S.B.H.
Trichoda (1)	B.H.
Enchylis (1)	B.H.
Urotricha (1)	B.H.
Uronema (1)	B.H.
Glaucoma (1)	B.H.
**Anoplophrya* (1)	B.
Paramoecium (2)	S.B.H.
Cyclidium (1)	S.B.H.
Cinetochilum (1)	B.H.
Heterotricha	
Spirostomum (1)	H.
Stentorella (1)	B.H.
Hypotricha	
Oxytricha (2)	B.H.
Stylonychia (1)	B.H.
Aspidisca (1)	S.B.H.
Peritricha	
Vorticella (2)	S.B.H.
Carchesium (2)	S.B.H.
Epistylis (1)	B.H.
Opercularia (2)	S.B.H.
Vaginicola (1)	B.H.
Suctoria	
Podophrya (1)	B.H.
Sphaerophrya (1)	B.H.

* Normally parasitic in enchytraeid worms but occasionally found free in the sewage.

S. = sedimentation tank, B. = bed, and H. = humus tank.

N.B. Figures in brackets indicate the number of species of each genus observed throughout the plant.

In the sedimentation tank the liquid has a high 'biochemical oxygen demand' and conditions are essentially anaerobic, but water movement and possibly rainfall can produce a temporary supply of dissolved oxygen especially near the surface. In the beds, conditions are of course aerobic because whatever the B.O.D. of the trickling liquid it is spread over the medium in a thin film. Conditions are stable and dissolved oxygen abundant in the humus tank. The influence of these environmental differences is shown in the number of forms and their abundance recorded in each case.

Conditions in the sedimentation tank favour few members of the phylum, while the rate of flow and the weekly cleansing are adverse to much multiplication of those able to maintain an anaerobic life, which contrasts with the findings of Lackey (1925 *a*, *b*) in the stabilized conditions of the Imhoff tanks. Flagellates form the most abundant section, but even these are relatively scanty. Rhizopods at no time exceeded 1000 per ml., while ciliates, with two exceptions, seemed incapable of surviving away from the unstable aerobic conditions at the surface. *Colpidium cucullus* Schrank and *Holophrya* sp. alone were commonly encountered away from the surface. Variations in the fauna would seem to be regulated by factors which are not constant and alter frequently, so that daily records made over a period or samples taken at different depths, with the exception noted, seem subject to no rule.

In the bacteria bed the number of Protozoa found is considerable, and records (Table 2) indicate marked variations in autumn and winter in the number of rhizopods and flagellates in contrast to spring and summer when they are fewer and more constant. Ciliates also show fluctuations of this nature, and this would suggest the influence on all of some obscure factors. Amongst the commonest genera represented in this environment are *Amoeba*, *Vahlkampfia*, *Arcella* and *Euglypha* in the rhizopods; *Monas*, *Oikomonas*, *Bodo*, *Cercobodo*, *Trepomonas* and *Pleuromonas* in the Mastigophora; and *Opercularia*, *Carchesium*, *Chilodon*, *Cyclidium*, *Urotricha* and *Cinetochilum* in the Ciliata. *Opercularia nutans* Ehr. is the dominant bed ciliate and shows interesting relationships with temperature. During the winter months low temperatures correspond to depression periods of *Opercularia*, whilst during the summer period temperature drops entail increased abundance. Coefficients of correlation between the numbers of *Opercularia* and temperature above and below 55° F. are not significant but have respectively the negative and positive sign and so indicate an optimum temperature of approximately 55° F.

Records for other individual forms also show seasonal variations. Some, such as *Amoeba limax* Duj., *Vahlkampfia*, *Cochliopodium*, *Monas*, *Oikomonas*, *Pleuromonas* and *Carchesium*, are represented throughout the year but show fluctuations with temperature similar to that of *Opercularia* described above; others, like *Bodo*, *Cercobodo*, *Anisonema*, *Chilodon* and *Urotricha*, are chiefly in evidence in winter. Forms especially abundant in spring, *Amoeba proteus* Leidy, *Arcella*, *Trepomonas*, *Cyclidium*, *Cinetochilum* and *Podophrya*, sometimes, but not always, have secondary maxima in autumn. Summer incidence is exhibited by *Cyathomonas*, *Lionotus* and *Uronema*, whilst *Trichoda*, *Oxytricha* and *Euglypha* are each in evidence in autumn. Since some forms appear both in spring and autumn and others only at one of these times it would appear that factors additional to temperature are responsible.

Similar successions to the above have also been noted in the humus tank. The stable conditions of this environment have proved more favourable for surveying the seasonal changes which are recorded for the three classes of Protozoa (Table 2) and for the individual forms (Table 3).

TABLE 2. *The seasonal abundance of Protozoa in the Knostrop beds and humus tanks*

Date 1939–41	Sarcodina				Mastigophora				Ciliophora				Temp. °F.	
	Bed		Humus		Bed		Humus		Bed		Humus			
	Liquid	Slide	Liquid	Slide	Liquid	Slide	Liquid	Slide	Liquid	Slide	Liquid	Slide	Bed Mean	Humus Mean
7 Dec.	—	—	40	20	—	—	—	—	—	—	70	800	36·3	—
14	1500	—	8	10	—	—	—	—	1600	—	8	400	43·0	—
21	—	—	4	20	—	—	—	—	—	—	60	1200	42·0	51·5
28	800	—	0	0	—	—	—	800	1100	—	20	1100	44·5	47·5
4 Jan.	—	—	20	—	—	—	—	—	—	—	80	—	39·0	49·0
11	1300	—	600	9	—	—	—	500	2300	—	50	200	41·9	49·0
18	—	—	300	0	—	—	—	400	—	—	20	100	37·7	47·5
25	1900	—	400	100	—	—	—	2700	1200	—	40	500	36·8	46·0
1 Feb.	—	—	100	0	—	—	—	300	—	—	8	0	36·5	—
8	400	—	4	0	13000	—	200	700	1000	—	0	200	33·3	44·0
15	—	—	30	50	—	—	900	800	—	—	200	100	35·2	45·0
22	2300	—	20	0	5000	—	300	900	3200	—	8	700	38·4	45·0
29	—	60	8	40	—	2300	0	800	—	200	10	800	43·5	48·0
7 Mar.	900	—	90	20	2200	—	200	600	1000	—	100	300	39·8	48·0
14	—	3100	300	30	—	2500	100	600	—	200	40	600	42·2	49·5
21	4600	—	70	60	3200	—	30	700	1900	—	40	1100	44·2	51·0
28	—	6500	70	20	—	3000	40	1100	—	1800	4	500	45·5	50·5
4 Apr.	1600	—	70	20	4100	—	200	600	2600	—	20	900	43·2	50·0
11	—	3400	100	0	—	1200	100	500	—	1800	70	800	46·5	51·5
18	600	—	300	—	500	—	300	—	1200	—	30	—	45·7	51·0
25	—	1700	200	50	—	800	90	1000	—	1100	8	1000	46·9	53·0
2 May	600	—	200	30	200	—	400	2400	500	—	40	2800	52·0	55·0
9	—	1200	20	20	—	400	200	700	—	600	20	4000	54·3	59·0
16	700	—	60	9	1400	—	400	4500	2300	—	40	1600	55·6	57·0
23	—	300	40	9	—	800	300	900	—	700	20	2400	56·1	60·5
30	1000	—	50	9	2200	—	300	300	1900	—	100	5300	56·9	62·0

6 June	—	700	30	0	—	1000	700	400	—	700	20	4700	67·9	63·5
13	700	—	100	20	300	—	200	400	1500	—	20	300	67·9	67·5
20	—	300	40	9	—	1500	200	700	—	2000	80	900	59·5	65·0
27	1400	—	40	9	1500	—	100	200	5600	—	400	2000	57·7	61·0
4 July	—	200	20	0	—	1000	400	200	—	1000	4	400	60·6	64·5
11	1000	—	30	—	200	—	200	—	3600	—	0	—	61·7	—
18	—	1700	100	9	—	1900	400	400	—	700	30	2600	60·1	62·0
25	1000	—	40	20	700	—	300	400	1700	—	300	700	58·3	62·0
1 Aug.	—	300	40	—	—	300	900	—	—	1000	200	—	62·8	65·0
8	100	—	50	—	200	—	800	—	500	—	20	—	66·5	—
15	—	300	50	—	—	700	400	—	—	600	50	—	61·7	61·0
22	1100	—	80	0	1300	—	400	500	10000	—	30	1500	58·6	62·5
29	—	800	70	0	—	1600	500	600	—	1000	80	2000	57·1	63·5
5 Sept.	800	—	80	0	900	—	300	500	1200	—	40	500	61·3	64·5
12	—	200	100	0	—	100	600	400	—	700	30	700	57·3	60·0
19	800	—	100	70	600	—	300	300	1500	—	50	700	55·0	59·0
26	—	1100	50	9	—	1000	200	400	—	1100	20	1300	53·0	59·5
3 Oct.	500	—	80	60	700	—	300	500	2100	—	20	1200	52·5	60·0
10	—	800	50	30	—	200	100	500	—	1000	100	2800	53·1	57·0
17	600	—	200	40	5600	—	1400	500	1400	—	40	1700	52·2	60·0
24	—	1000	200	9	—	2700	300	500	—	600	60	2500	51·7	57·5
31	800	—	30	20	1300	—	1000	600	5700	—	40	3500	45·5	53·0
7 Nov.	—	1000	20	20	—	3200	200	500	—	2700	20	4700	47·7	54·0
14	1900	—	20	9	3900	—	100	300	2200	—	50	400	46·0	—
21	—	2000	0	30	—	1100	200	300	—	300	8	800	46·5	54·0
28	400	—	40	9	1900	—	400	700	1600	—	40	3900	46·3	52·5
5 Dec.	—	5300	—	90	—	5400	—	600	—	1800	—	7200	46·3	53·0
12	—	—	—	—	—	—	—	—	—	—	—	—	43·7	—
19	—	1500	—	—	—	2500	—	—	—	800	—	—	44·7	—
26	—	—	—	—	—	—	—	—	—	—	—	—	42·5	—
2 Jan.	—	2700	—	—	—	2900	—	—	—	3800	—	—	43·6	—
9	—	—	—	—	—	—	—	—	—	—	—	—	38·1	—
16	—	2300	—	—	—	3700	—	—	—	1900	—	—	38·6	—
23	—	—	—	—	—	—	—	—	—	—	—	—	29·0	—
30	—	1100	—	—	—	1500	—	—	—	500	—	—	32·0	—

Liquid: organisms in numbers per ml. Slides: Organisms in numbers per sq. cm.

TABLE 3. *Organisms showing seasonal changes in abundance as recorded in numbers per sq.cm. occurring on the humus slide*

Date 1939–40	*Arcella*	*Euglypha*	*Bodo*	*Trepomonas*	*Cyathomonas*	*Chilodon*	*Cyclidium*	*Oxytricha*	*Cinetochilum*	*Uronema*	*Paramoecium*	*Stentorella*	*Trichoda*	Mean temp. °F.
7 Dec.	—	9	—	—	—	54	9	—	—	9	—	—	—	—
14	—	18	—	—	—	18	—	9	14	—	—	—	—	—
21	18	—	—	—	—	9	—	—	—	—	—	—	—	49·5
28	—	—	50	—	—	20	9	9	14	—	—	—	—	47·8
4 Jan.	Tank cleaning sequence broken and record not available													
11	—	9	—	—	—	36	—	—	—	—	—	—	—	48·4
18	—	—	—	—	—	73	—	—	—	—	—	—	—	47·9
25	134	—	237	—	—	182	—	—	—	—	—	—	—	46·6
1 Feb.	—	—	45	—	—	—	—	—	—	—	—	—	—	—
8	—	—	23	—	—	162	—	—	—	9	—	—	—	44·7
15	27	—	23	—	—	9	—	—	—	—	—	—	—	44·0
22	—	—	67	—	—	491	—	—	—	—	—	—	—	45·0
29	9	—	135	—	—	418	27	—	—	—	—	—	—	45·7
7 Mar.	18	—	23	—	—	300	—	—	—	—	—	—	—	47·5
14	18	—	45	—	—	218	9	9	9	—	18	—	—	49·0
21	—	—	45	—	—	782	36	9	—	—	—	—	9	50·6
28	—	—	114	—	—	155	9	9	—	—	—	—	9	51·4
4 Apr.	18	—	23	—	—	109	9	9	—	—	—	—	—	50·2
11	—	—	18	—	—	9	18	36	27	—	27	—	9	51·2
18	Tank cleaning sequence broken and record not available													
25	18	—	9	—	—	291	9	9	—	—	—	—	—	51·7
2 May	27	—	91	—	55	18	—	—	—	—	9	—	—	53·0
9	—	—	45	—	—	146	—	9	27	—	—	—	—	58·0
16	9	—	91	—	—	100	—	82	18	—	—	—	27	58·7
23	9	—	—	—	27	109	9	9	—	—	—	—	—	59·5
30	9	—	—	—	18	—	—	9	9	—	9	—	—	62·1

6 June	—	—	—	—	—	—	—	—	27	—	—	—	—	63·3
13	—	—	46	—	—	—	—	9	—	—	—	—	—	67·0
20	9	—	—	—	—	18	9	—	9	73	36	—	—	65·7
27	9	—	—	23	18	—	—	—	—	55	45	45	—	61·5
4 July	—	—	—	23	27	—	—	—	—	—	9	—	—	63·8
11	Tank out of action owing to low volume of sewage													
18	9	—	—	—	—	—	—	9	9	—	9	45	—	63·5
25	9	—	—	23	18	—	—	—	—	—	18	45	18	62·5
1 Aug.	Tank out of action owing to low volume of sewage													
8														
15														
22	—	—	23	9	45	—	—	—	—	—	18	9	—	62·5
29	—	—	23	—	—	18	—	9	9	45	82	9	27	62·0
5 Sept.	—	—	91	45	27	—	—	—	—	—	127	—	—	62·5
12	—	—	—	45	45	—	—	—	—	—	91	—	—	59·0
19	—	—	23	—	9	—	—	—	—	—	—	—	—	57·5
26	9	—	32	9	—	—	9	9	—	9	27	18	—	57·5
3 Oct.	9	—	23	—	—	—	—	—	—	—	—	9	9	56·0
10	9	—	—	—	—	18	—	9	—	—	73	27	45	58·5
17	9	—	—	—	—	—	9	27	18	—	9	18	36	58·5
24	—	—	23	—	—	—	—	9	—	—	155	18	9	58·0
31	—	16	64	—	36	—	—	—	—	—	—	27	9	54·0
7 Nov.	9	9	—	—	—	—	—	9	—	—	—	27	9	52·5
14	9	—	27	—	—	9	—	—	18	—	—	—	—	49·0
21	—	—	—	—	—	—	9	—	—	—	18	—	—	52·5
28	—	9	45	—	23	—	—	9	9	—	18	—	—	55·0
5 Dec.	18	—	32	—	—	45	—	18	—	—	9	18	—	52·0

N.B. Correlations of abundance with temperature given in the text are calculated using Fisher's (1928) formula.

The fauna of the channels between the bacteria bed and humus tank and beyond the humus tank resembles, both in variety and abundance, that found in the humus tank itself, thus showing that there is no appreciable increase in the latter. Similarly, no increase was noted in the tank over the period of a week's operation, and it would seem therefore that the rate of flow and circulation in the humus tank is unfavourable to the accumulation of Protozoa. Many types settle on slides suspended in the liquid and multiply there, so that other conditions in the tank must be favourable. These slides do not give a real estimate of the circulating abundance, but they do emphasize the seasonal succession of certain forms, particularly the representatives of the Holotricha, Heterotricha and Hypotricha, whilst the

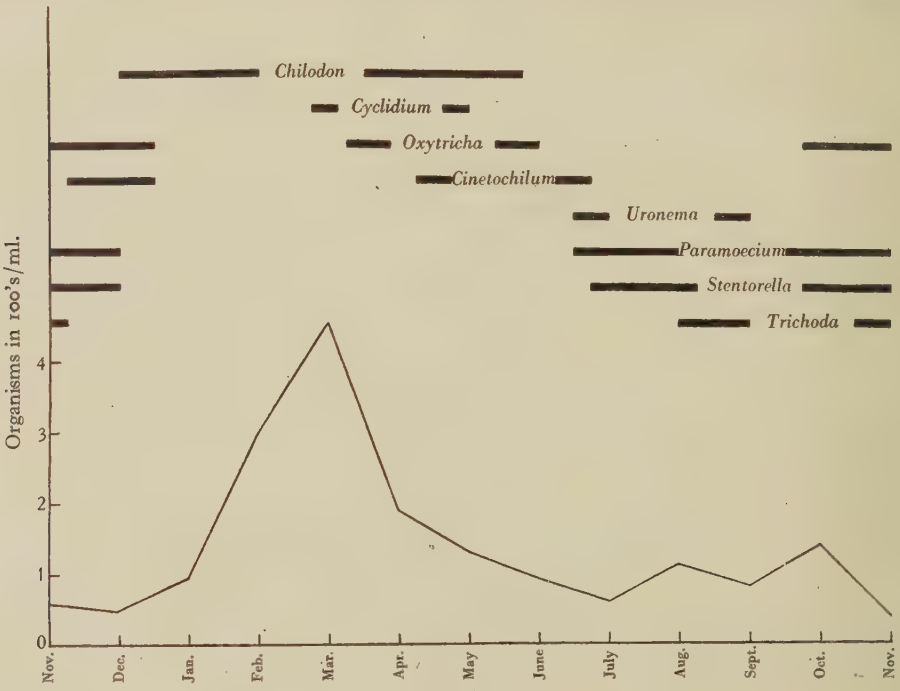

Fig. 1. Seasonal succession and abundance of free-swimming ciliates on the humus tank slides.

Peritricha show a relationship with temperature. It is difficult to say whether the presence or absence of many of the free-swimming ciliates is dictated by physical factors or by biological competition, but since equivalent temperatures do not always support the same forms it seems likely that both are important.

Abundance and seasonal succession of many forms are correlated with temperature (Table 3). *Chilodon*, which is present in winter to such an extent that its numbers are greater than those of all the free-swimming ciliates found in summer (Fig. 1), shows a negative correlation ($n=40$, $r=-0{\cdot}375$, $P=0{\cdot}02$) as also does *Arcella* ($n=26$, $r=-0{\cdot}49$, $P=0{\cdot}01$) (Fisher, 1928). *Cyathomonas* and *Trepomonas* show summer incidence and positive correlations with temperature ($n=40$, $r=+0{\cdot}42$, $P=0{\cdot}01$ and $n=40$, $r=+0{\cdot}36$, $P=0{\cdot}02$ respectively). *Cyclidium* has an optimum temperature between 50 and 52° F. based on

calculations of the correlation coefficient between abundance and temperature (over 50° F., $n=26$, $r=-0{\cdot}556$, $P=0{\cdot}01$ and below 52° F. $n=13$, $r=+0{\cdot}827$, $P=0{\cdot}01$) and *Carchesium* has a similar correlation to that of *Opercularia* in the bed. Forms which do not correlate with temperature but which are seasonal in occurrence are *Trichoda*, *Euglypha* and *Stentorella* which are again in evidence in autumn, *Oxytricha* and *Cinetochilum* in spring and autumn, and *Paramoecium* in summer and autumn. All the ciliates provide a succession of forms throughout the year (Fig. 1) resembling the succession of *Aspidisca*, *Colpoda* and *Lembus* recorded by Lackey (1924), which he attributes to variations in food requirements. He considers that *Opercularia* occurs independently of the other forms and possibly requires a different type of nutrition from the free-swimming ciliates.

Discussion

This investigation was undertaken to obtain some knowledge of the Protozoa inhabiting sewage purification works from the point of view of their ecology in this environment. Such information is also of value since their presence may have practical significance in relation to the efficiency of the various processes in the works as in the case of the Enchytraeidae (Reynoldson, 1939 *a*, *b*) and Insecta (Lloyd, 1935). A wide variety of forms was encountered, and as would be expected, many of the Protozoa inhabiting fresh water have gained access to the plant.

Once they arrive at the works the Protozoa find themselves in the highly anaerobic conditions of the sedimentation tanks. In this environment only true anaerobic forms could thrive, but many others may survive the passage through owing to the false aerobic conditions found at the surface or after heavy rainfall; others may pass through in the encysted state. The tanks are emptied weekly, and the constant flow of water allows no time for a distinctive fauna to establish itself or exert any real influence upon the water. In contrast, the liquid in the Imhoff tanks is not constantly changing so that the fauna suffers little disturbance. Lackey (1925 *a*) lists sixty-four species of Protozoa from the Imhoff tanks, of which twenty-nine are described as common and the rest as rare or in passage, whilst thirty species have been recorded here of which thirteen were common (two rhizopods, nine flagellates and two ciliates). *Holophrya* sp. (noted also by Lackey) and *Colpidium cucullus* are the only common ciliates, and they are found at the surface and deep in the tank. *Trepomonas agilis* Duj. and *Hexamitus inflatus* Duj. are common in the Imhoff tanks and *Polytoma uvella* Ehr. is rare, but the first two are rare and the latter common in the sedimentation tanks. These differences are no doubt associated with the absence of an established fauna in the sedimentation tanks, and in confirmation of this, it seems that some time elapsed before the characteristic fauna was established in the Imhoff tanks on those occasions when they were refilled (Lackey, 1925 *b*) and there was very little increase in the number of organisms till after the first 3 weeks (Lackey, 1924).

The sewage from the sedimentation tanks is discharged on to the bed by means of sprays and is therefore aerated by agitation. It passes through the bed in the form of a thin film, providing a favourable environment for aerobic Protozoa in any part of the bed irrespective of the B.O.D. of the sewage: eleven rhizopods, eighteen flagellates and thirty-four ciliates were found to be more or less common near the bed surface. A greatly increased proportion of ciliates, especially *Vorticella*, *Carchesium* and *Opercularia*, which was the dominant species, occurred, compared with the sedimentation tanks. Johnson's (1914) list of the bed

Protozoa included many of these forms, but *Epistylis* apparently replaced *Opercularia* which was not included.

Barritt (1940) found that in a poor or young activated sludge, the Protozoa are varied but lack the Peritricha, while the latter abound to the almost complete exclusion of other forms in an old efficient sludge. Forms characteristic of both kinds of sludge occur in the bacteria bed. Further, seasonal changes would not seem to affect the equilibrium of the ciliates in the bed since the Peritricha are abundant throughout the year, and the seasonal succession of the other ciliates ensures a varied fauna throughout both summer and winter. Similar though less marked successions are present in the rhizopods and flagellates.

Protozoa washed out of the bed pass through the humus tank and, as in the case of the sedimentation tank, the rate of flow restricts multiplication of forms. The relationship between the number of forms washed out of the bed and the actual number of forms recorded from the bed slides has been considered, but although there are some indications of a correlation, a definite association was not established.

Seasonal succession of the free ciliates is shown on slides suspended in the humus tank, and it is found that *Carchesium* replaces *Opercularia* as the dominant form. This change may be due to the greater ease with which dispersal and recolonization are effected by *Carchesium* since both *Carchesium* and *Opercularia* are found in large colonies in the bed while *Carchesium* alone forms large colonies on the slides in the humus tank. The peritrichous forms in general are to be found in almost every sample but show certain fluctuations with temperature.

In conclusion, it can be stated that there are definite fluctuations in abundance and biological succession of the protozoan fauna which are related to the physical and chemical conditions of the environment. These fluctuations are sporadic in the sedimentation tanks and dominated by erratic changes in the character of the sewage, but in passing through the bacteria beds to the humus tanks seasonal influences become increasingly evident as a result of the increased purity and consequent uniformity in the composition of the sewage. Further, while some of these changes are linked with temperature others are more elusive and may be due to biological factors such as changes in the character of the food supply or to competition.

The extent to which the Protozoa have an essential or effective role as regards the efficiency of the bed has yet to be determined, but this survey should make a useful basis for such investigations.

Summary

The protozoan fauna of the bacteria bed process of sewage purification has been studied through a year and compared with that of the Imhoff digestion tanks and of the activated sludge process. The anaerobic water of the sedimentation tanks recalls the conditions of the Imhoff tanks, but the movement of water and cleaning prevents the establishment of a strongly characteristic fauna. Similarly, movement and cleaning in the humus tank does not allow the development of strong characters, and the species found are evidently all from washings of the filter beds. In the latter, rhizopods, flagellates and ciliates abound and species dominant in young inefficient and old efficient activated sludge are intermingled. Fluctuations of the fauna of the sedimentation tanks appear to be dominated by sporadic

change in the character of the sewage, but in the bed and humus tanks seasonal variations occur in all forms but are most evident in the Ciliata. Changes in abundance occur in the peritrichous forms, and in other ciliates a seasonal succession of species is noted. The occurrence or abundance of some forms can be linked with temperature but in a few the appearance is either vernal or autumnal with equivalent temperatures and so is due to some more obscure factor such as nutrition or competition.

My thanks are due to Dr Ll. Lloyd for his invaluable advice and assistance especially in relation to the identification of the forms recorded, and to Mr J. T. Thompson of the Leeds Corporation Sewage Works for permission to visit the Knostrop Works and for supplying data relating to the plant. I also wish to thank Prof. E. A. Spaul for his suggestions during the investigations and for his help in the preparation of the manuscript.

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PRELIMINARY INVESTIGATIONS ON THE VALUE OF ELECTRIC HEATING OF BEEHIVES

By C. G. BUTLER and G. F. COCKBILL
Bee Department, Rothamsted Experimental Station, Harpenden

Preface

This report describes the methods and the results obtained in certain preliminary experiments conducted in the Home Apiary of the Rothamsted Experimental Station Bee Department during the winter 1940–1 in collaboration with Electrical Research Association, with an electrical heating device (Wedmore, 1941) designed for use with beehives.

Kalabuchov (1934) showed that the honeybee dies very quickly at temperatures between 1 and 8° C., death being attributed to a differential effect of cold on the various steps in the utilization of sugar. At 1° C. metabolism in the tissues still goes on actively, but absorption from the gut is arrested and, since the honeybee is dependent on sugar in its diet, it soon dies of starvation at low temperatures. Actually, the honeybee colony in winter possesses a remarkable degree of temperature regulation. As the mean temperature falls about October, breeding, which requires a relatively high temperature (34–35° C.), is greatly reduced and ceases altogether about the beginning of December, when the bees commence to form a more or less compact cluster which furnishes considerable thermal protection. According to Schulz (1927) the temperature of the cluster passes through a series of cycles (Lammert's cycles). When the temperature of the outside of the cluster falls to 13° C. the bees on the surface are stimulated to muscular activity, thus causing a rapid rise in cluster temperature to a maximum of 24–25° C. which then gradually falls again over a period of hours until a temperature of 13° C. is reached once more, when the cycle is repeated. Once the temperature has risen muscular activity ceases for the time being. It is estimated that an average-sized colony consumes about 20 g. of sugar, yielding 80 cal., in order to produce the necessary heat during a single cycle which according to Schulz (1927) lasts about 22–23 hr.

Unfortunately, inaccessibility of the data on which Lammert & Schulz (1927) based their belief in this cycle of 22–23 hr. makes it impossible to reach any conclusion on its reliability, and, as no effects of such cycles were noticeable in the present work, this factor has been ignored.

From the above it would appear that some form of artificial heat, applied in a satisfactory manner, should reduce the consumption of stores by preventing the temperature dropping to as low as 13° C., or at any rate slow down the rate of loss of heat. It should also, by removing the necessity for the consumption of so great a bulk of stores during a prolonged cold spell, prevent the hindgut of the individual bee becoming unduly filled with indigestible material at a time when a cleansing flight is impossible. It is stated by Phillips & Demuth (1914) that the bees in a winter cluster are most quiescent when the cluster temperature is between 14 and 20° C. This is not in full agreement, at any rate as regards the lower temperature, with Lammert's hypothesis (Schulz, 1927). However, the most desirable temperature of the air inside the hive at which to maintain a colony of bees throughout the broodless period of winter probably lies somewhere between these limits. If it can be found

and maintained, the saving of stores and prolongation of the life of the bees may prove to be an economic proposition.

In the early part of the year, generally towards the end of January, brood rearing commences once more and the much higher temperature of 34–35° C. has to be maintained constantly in that part of the colony where the brood is present. Thus, if the winter is cold, considerable quantities of stores are consumed. Further, if a cold spell intervenes towards the beginning of April when the mean daily temperature has increased and the amount of brood present is expanding rapidly, the colony receives a serious check and, in severe cases, the bees are compelled to cluster once more and the brood that they have been forced to desert becomes chilled and dies. Under such circumstances it would seem likely that the application of adequate heat in the spring would prove beneficial and help to stimulate the building up of strong colonies for pollination of early fruit and other crops. The production of really strong colonies in the early spring for this purpose would appear, according to the work of Woodrow (1933) and Filmer (1932), to be of considerable importance, since an increase in colony strength results in a proportionately greater increase in the number of available foraging bees, e.g. a doubling of colony population leads approximately to a fourfold increase in the number of foraging bees. A further important point is that electrical heating might be expected to assist ventilation and the removal of the excess water vapour given off by the bees in their metabolism and thus help to reduce the indirect losses caused by condensation of moisture in the colder parts of the hive.

Experiments on the electrical heating of beehives have already been conducted in Germany by Bardenbacher (1937), who reported very promising results, suggesting that brood raising may be advanced by about 3 weeks by appropriate heating on a moderate scale in the spring season, and that one such advanced colony may well do the work of two colonies of average strength. The primary object of the experiments about to be described was to test the effect of different forms of winter and spring heating on the strength of colonies at the beginning of the next season. It was also intended to study the temperature changes within the hives in relation to the temperature outside.

Methods

In the first instance experiments were conducted with a well-lagged heating unit arranged to fit directly over the top of the brood chamber. The unit was constructed with the dimensions of a 6 in. deep lift to fit on top of the National type of hive, using the unit itself as a crown-board. The heating was arranged to give about 9–10 W. by means of two 15 W. 230 V. lamps run in series. Hot spots due to the heating elements were eliminated by the use of a metal plate to distribute the heat, plus a small baffle plate immediately beneath each heating unit. By using the unit as a crown-board the difficulty of overheating the individual combs by conduction was overcome. Most of the heat is thus transmitted by radiation and some by convection: the under-surface of the metal plate was painted dull black to enhance radiation. The metal plate bed, besides having a central feed hole protected with perforated zinc, had a 1 in. diam. ventilation hole in each corner. The whole unit was filled to within about 1 in. of the top with dry sawdust as lagging to prevent the escape of heat.

Thirty colonies of bees housed in National single-walled hives, each containing ten (instead of the customary eleven) B.S. frames were selected for these experiments in September 1940. The amounts of brood and adult bees in each hive were equalized as far as possible, and each colony had abundant stores of pollen and 30–35 lb. of sealed stores consisting largely of honey.

In order to ensure, as previously found desirable for good wintering, thorough ventilation and the consequent removal of water vapour produced by the metabolism of the bees, no entrance blocks were used, full entrance being given in each case. The hive entrances were protected against mice with perforated zinc, an exit for the bees 3 in. long and about $\frac{1}{4}$ in. high being left in the centre. The

full entrance together with the ventilation holes in the metal plate of the heating unit and a deep roof ensured complete dryness of the brood-chamber at all times, an essential point if satisfactory wintering is to be achieved.

The thirty colonies selected for the experiments were divided into five groups of six. The first group (T_A) had the heating units thermostatically controlled and were so wired that the electrical current was switched on and heat generated when the temperature in a Stevenson screen in a fully exposed part of the apiary fell below 6·5° C. The second group (P_A) had similar heating units, supplying heat at the same rate, but permanently in operation. These two groups had their heat turned on from 16.00 hr. G.M.T. on 15 October 1940. Two further groups (T_S, P_S) were similarly equipped, but were only heated in the spring, and thus, prior to the turning on of their heat at 10.00 hr. G.M.T. on 1 February 1941, were under similar conditions to the six control colonies (C) which had no artificial heating at all, but had empty shallow chambers filled with sawdust placed on top of the brood chambers in order to make them comparable in regard to packing, with the experimental colonies. Each group of six colonies was further subdivided into two with old (1939) queens and four with young (1940) queens.

Unfortunately, on account of difficulties in wiring the apiary, it was impossible to satisfy the usual requirements of experimental design by assigning the treatments randomly to the available colonies. The thirty colonies had been balanced as carefully as possible so as to be of approximately equal strength in every respect, and there is thought to be little likelihood of any position effect biasing treatment comparisons as a result of systematic arrangement of the experimental colonies.

Temperature readings within the hive were obtained by means of ten copper-constantin thermocouples, the warm junction of one being embedded centrally in the comb of each frame (i.e. 4½ in. below the top bar and 7¼ in. from each side bar). The cold junctions of the ten thermocouples from the frames in each hive brought outside the back were bound together, and placed in a Thermos flask containing ice and water at 0° C. The leads from each thermocouple were soldered to an ordinary brass two-pin socket; leads could then at will be connected to any particular thermocouple and carried up to the apiary hut by means of insulated flex. The temperature of the warm junction could be read by means of a calibrated micro-milliammeter, the current being balanced by means of a potentiometer circuit incorporating a mirror galvanometer. Individual readings with this apparatus were accurate to within 0·4° C.

Only twenty-five colonies could be equipped with thermocouples, and one hive (always with a young queen) from each heating category was omitted from this aspect of the experiment. Until mid-December, temperatures of each frame of these twenty-five colonies were recorded almost every morning; when regular observations were resumed after Christmas, an interval of 2–3 days was generally adopted until the end of March, after which observations were discontinued. It took about 1½ hr. to take all the temperatures. At the beginning and end of each set of observations the temperature inside the Stevenson screen was also recorded by means of a thermocouple.

Results and conclusions

(1) *Results of heating.* On 16 Apr. 1941 the thirty colonies concerned in these experiments were examined and estimates made of the area of brood in each. It was assumed that each 'patch' of brood was approximately elliptical in shape, and rapid measurement of its length and breadth was made in order to estimate the area. Two colonies (one with thermostatically controlled heat turned on in February 1941 and having an old queen, the other permanently heated from February 1941 and having a young queen) had to be rejected on account of queen failure, and damage caused by mice, respectively. The means which follow have been adjusted to take account of this.

There was very great variability between colonies in their total area of brood, one treatment (T_A, young queen) having areas 701, 490, 427 and 104 sq. cm. Hence only large effects of treatments could possibly be demonstrated as significant with the degree of replication in the experiment. The only treatment to show any sign of a beneficial effect was P_A, and its excess over the control hives is well within the margin of variability of the material (Table 1). Three of the treatments (T_A, T_S and P_S) appear actually to have depressed the area of brood produced as compared with the unheated hives. There are undoubtedly

significant differences between the five groups of six hives, but it is not easy to find a satisfactory explanation of the situation. The average of all four types of heating is less than the control, though scarcely significantly so. It is, however, demonstrated that no great benefit resulted from the heating. Old and young queens gave means in very close agreement.

The weight of stores remaining in each hive on 17 Apr. 1941 was also obtained. Just before the beginning of the experiment the total sealed stores of each colony had been made up as nearly as possible to 35 lb. by feeding sugar syrup (2:1 weight). Table 2 shows the consumption of winter stores under the various treatments. Again the significant differences between heating treatments are not easy to explain. It is apparent that the colonies that were constantly heated throughout the winter (P_A) consumed the least stores and gave the most brood, whereas those having the other three types of heat consumed more stores—on the average, significantly more—than the control. There is also a difference between colonies having young and old queens which is just significant, the latter consuming 2·2 lb. of stores more than the former.

TABLE 1. *Mean brood areas on 16 Apr. 1941 (in sq. cm.)*

	T_A	P_A	T_S	P_S	C	Mean
Old queen	473	760	534	445	548	552±49
Young queen	430	679	474	439	650	535±35
Mean (±63)	445	706	494	441	616	540

TABLE 2. *Consumption of stores (lb. per hive)*

	T_A	P_A	T_S	P_S	C	Mean
Old queen	32·0	27·0	32·0	33·5	29·5	30·8±0·85
Young queen	29·0	25·8	30·8	30·0	27·2	28·6±0·60
Mean (±1·09)	30·0	26·2	31·2	31·2	28·0	29·3

TABLE 3. *Number of frames of adult bees*

	T_A	P_A	T_S	P_S	C	Mean
Old queen	6·0	5·5	5·5	3·5	4·8	5·0±0·28
Young queen	4·2	4·6	4·9	3·9	4·0	4·3±0·20
Mean (±0·37)	4·8	4·9	5·1	3·8	4·2	4·6

On 17 Apr. estimates were also made of the number of brood frames occupied by adult bees (Table 3). There are no significant effects of heating, although all treatments except P_S showed more frames occupied than the control hives. The difference between the number of frames occupied by adult bees in colonies containing old and young queens, 0·7 frame, was almost significant.

(2) *Temperatures within the colonies.* Three functions of the temperatures recorded for each colony were examined and analysed. The mean of the figures on any one occasion from the ten frames may be taken as representative of the average conditions within the colony. The highest of the ten temperatures is an approximation to the temperature at the centre of the winter cluster of the bees—presumably an underestimate of the true figure, but probably not very far from it. The lowest of the ten frame temperatures, which generally occurred at one or other of the two extreme positions (i.e. outside combs), represents conditions fairly well removed from the bees and is indicative of the purely physical temperature changes inside a hive.

For the subsequent analysis the temperatures were divided into six periods. The first covers a preliminary series of observations before the heating was begun, from 9 to 14 Oct. 1940. The other five are each of a month's duration, 17 Oct.–15 Nov., 16 Nov.–14 Dec., 1–31 Jan., 1–28 Feb. and 1–31 Mar. The last fortnight of December has been omitted, since it contained a week in which no temperature observations were made.

The mean temperatures of the ten frames for each of the periods are shown in Table 4. The screen temperatures shown are average readings for the mornings on which the observations were made (generally between 08.00 and 11.00 hr. G.M.T.). Except for the last two periods (i.e. before the heating was applied to them on 1 Feb. 1941) T_S and P_S are effectively additional control treatments.

TABLE 4. *Mean hive temperatures* (° C.)

	Preliminary	Oct.–Nov.	Nov.–Dec.	Jan.	Feb.	Mar.
T_A	17·5	18·8	15·2	15·8	16·9	16·9
P_A	19·3	16·9	13·6	12·3	13·3	17·3
T_S	18·0	15·8	10·2	10·8	15·7	17·2
P_S	17·7	13·5	10·1	7·6	12·8	15·8
C	17·7	14·3	10·4	9·8	12·2	16·0
S.E.	±0·89	±0·79	±0·92	±1·58	±1·38	±0·87
Mean	18·0	15·9	11·9	11·3	14·2	16·6
Screen	11·3	8·1	5·0	0·0	1·9	4·2

It is clear that the colonies having thermostatically controlled heating applied through the winter (T_A) were consistently at a higher mean temperature than the controls until February, during which month the difference decreased; by March it had almost disappeared. The P_A (constantly heated throughout the winter) was also significantly warmer than the controls, but showed surprisingly lower temperatures than the T_A (thermostatically controlled heat throughout winter), though this difference was scarcely significant. Those colonies which had thermostatically controlled heat applied from February onwards (T_S) appeared to increase their temperatures and approximate to the T_A, though their excess over the controls was not significant. There was little indication of any gain in mean temperature by the P_S colonies (constantly heated from 1 Feb. onwards).

It was hoped that the temperatures for the preliminary period might be used in a covariance analysis in order to reduce the variability of colonies under the same experimental treatment by removing components attributable to initial differences. Examination of the records for individual colonies, however, indicated that there was very little association between the preliminary and experimental mean temperatures, even for the first month of the observation.

A second form of analysis was undertaken in order to correlate hive temperature with the corresponding screen temperature. If such a correlation or regression analysis is made uncritically on the data as they stand, the effects which are of real importance may well be masked by 'long-term trends'. Both hive and screen temperatures show, apart from day-to-day irregularities, a general tendency to decrease in the first half of the winter and later show general increases, but such a seasonal cycle is a well-known phenomenon and the association between hive and screen in this respect is of little interest. On the other hand, there are very considerable departures from regularity in these temperature trends, and real importance attaches to the association between corresponding departures from regularity in hive and screen temperatures.

The elimination of long-term trends from data such as the present may be accomplished in several ways. The method used here was to correlate changes in hive temperature between successive days of observation with the corresponding changes in screen temperature, i.e. to correlate first differences of the series of observations. This, though simple in operation, is open to objection on statistical grounds in that the separate observations do not enter the analysis independently and the standard errors of estimates obtained are of doubtful validity. Much of the data was, however, initially analysed in this way and it was felt that the results were sufficiently clear to make it preferable to complete the examination in the same form rather than to recompute by an alternative method.

In this way regression coefficients may be obtained estimating the average change in hive temperature resulting from an increase of 1° C. in screen temperature. Such regression coefficients have been computed separately for the average of all hives on a given heating treatment for each of the first three experimental months and for the last two together. For

TABLE 5. *Estimated increase in* °C. *in mean hive temperature for a rise of* 1° C. *in screen temperature*

Treatment	Before turning on spring heat	After turning on spring heat
T_A	0·004 ± 0·062	0·039 ± 0·091
P_A	0·263 ± 0·073	0·187 ± 0·141
T_S	0·370 ± 0·073	0·061 ± 0·102
P_S		0·483 ± 0·179
C		0·245 ± 0·154

TABLE 6. *Highest hive temperatures* (°C.)

	Preliminary	Oct.–Nov.	Nov.–Dec.	Jan.	Feb.	Mar.
T_A	22·7	28·5	25·7	29·3	31·7	31·4
P_A	26·3	23·2	19·8	23·2	25·6	29·4
T_S	25·2	21·9	15·9	21·7	28·0	29·2
P_S	25·0	20·2	16·4	18·0	26·5	29·0
C	24·6	19·9	15·4	21·3	25·5	29·1
S.E.	± 1·62	± 1·37	± 1·72	± 3·00	± 2·30	± 1·69
Mean	24·8	22·7	18·6	22·7	27·5	29·6
Screen	11·3	8·1	5·0	0·0	1·9	4·2

simplicity of presentation of the results, the sixty-three temperature changes for the first 3 months have been put together as there were only small differences between the separate results, and the regression coefficients for these and for the twenty-three changes obtained in February and March are shown in Table 5. For the first set the T_S and P_S colonies have been included with the controls.

The colonies whose heating was thermostatically controlled, whether throughout the winter or only in the spring, had a mean temperature whose fluctuations were independent of those of the temperature outside. The control colonies varied in accordance with external fluctuations, increasing by about 0·3° C. for a rise of 1° C. in the screen temperature, and the behaviour of the continuously heated colonies was similar. Differences between the regression coefficients for any one period cannot be tested by means of the standard errors given in Table 5; it may be shown that the P_A colonies were significantly less dependent on the outside temperature changes than were the controls during the period of the first observations.

As with the mean hive temperatures, the highest hive temperatures tabulated in Table 6

show the most extreme behaviour by the T_A series. During the preliminary period differences between the five groups were small, and indeed the T_A was the lowest, but after the turning on of the heat on 1 Feb. the hives of this group maintained an advantage of 8–10° C. over the controls until the latter began to increase their highest temperatures in February. In the early months the P_A colonies were higher (though not significantly so), than the controls, but the spring heating did not appear to produce any effect.

Table 7 shows regression coefficients for the highest hive temperatures analogous to those of Table 5 for the mean temperatures. Its most noticeable feature is that in every case a rise in screen temperature was associated with a fall in the highest temperature of the ten frames, suggesting a reduction in the activity of the bees. The magnitude of this effect tended to be greater with the artificially heated hives than with the controls, though the differences are scarcely significant and are reversed in sign by the P_S group. There was an average reduction of about 0·2° C. for each 1° C. increase in the outside temperature.

TABLE 7. *Estimated increase in* °C. *in highest hive temperature for a rise of* 1°C. *in screen temperature*

Treatment	Before turning on spring heat	After turning on spring heat
T_A	−0·302 ± 0·079	−0·181 ± 0·057
P_A	−0·281 ± 0·101	−0·375 ± 0·113
T_S	−0·144 ± 0·093	−0·204 ± 0·069
P_S		−0·008 ± 0·150
C		−0·175 ± 0·123

TABLE 8. *Lowest hive temperatures* (°C.)

	Preliminary	Oct.–Nov.	Nov.–Dec.	Jan.	Feb.	Mar.
T_A	12·4	11·5	7·6	5·3	5·8	8·1
P_A	13·6	11·8	8·7	5·0	5·8	9·2
T_S	11·8	10·1	5·8	2·7	6·2	8·5
P_S	12·5	9·1	6·0	2·5	6·0	9·3
C	13·1	9·8	6·1	2·6	4·2	7·2
S.E.	±0·39	±0·38	±0·28	±0·43	±0·51	±0·40
Mean	12·7	10·5	6·8	3·6	5·6	8·5
Screen	11·3	8·1	5·0	0·0	1·9	4·2

Table 8 shows the means of the lowest temperatures of the ten frames in a colony. Both the T_A and P_A groups were consistently higher than the controls, the difference being generally clearly significant. When the spring heating was begun the T_S and P_S colonies rose to much the same minimum temperature as the other heated groups. The controls were about 1–3° C. higher than the outside temperature throughout.

As might be expected the dependence of lowest hive temperatures on screen temperature was considerably closer than that of mean or highest hive temperature. The regression coefficients are shown in Table 9. The thermostatic control succeeded in damping this dependence to some extent, a rise of 1° C. in outside temperature being associated with a rise of 0·4° C. in a thermostatically controlled hive. With continuous heating or in an unheated hive the corresponding increase was 0·6–0·7° C. The figures for the early and later winter were in remarkably close agreement and there was no indication of any difference between the effects of autumn and spring heating.

Differences in temperature between colonies having old and young queens are summarized in Table 10. In no case was there a significant difference, but the colonies with old queens were almost always slightly warmer in every respect. As the differences were so small, no attempt was made to estimate separate regressions on screen temperature for the two classes of colony.

Finally it may be noted that there was no indication of association between any of these temperature measurements and the areas of brood in individual colonies.

TABLE 9. *Estimated increase in* °C. *in lowest hive temperature for a rise of* 1°C. *in screen temperature*

Treatment	Before turning on spring heat	After turning on spring heat
T_A	0·403 ± 0·083	0·388 ± 0·103
P_A	0·602 ± 0·080	0·637 ± 0·165
T_S	0·688 ± 0·060	0·384 ± 0·132
P_S		0·730 ± 0·179
C		0·643 ± 0·155

TABLE 10. *Differences in temperatures, old-young queen colonies* (°C.)

Period	Hive temperature: Mean		Highest		Lowest	
Preliminary	0·46	± 0·92	− 1·15	± 2·08	0·33	± 0·32
Oct.–Nov.	0·79	± 0·75	− 0·59	± 1·30	0·12	± 0·35
Nov.–Dec.	1·32	± 0·86	0·10	± 1·62	0·56	± 0·26
Jan.	1·43	± 1·50	0·91	± 2·83	0·49	± 0·41
Feb.	2·00	± 1·26	0·28	± 2·10	0·77	± 0·47
Mar.	1·21	± 0·80	1·06	± 1·54	0·40	± 0·41

SUMMARY

A set of thirty colonies of honeybees was divided into five groups, four of which received different types of artificial heating during the winter of 1940–1. The general effect of heating the colonies appeared in these preliminary experiments to be to reduce the area of brood present in mid-April and to increase the winter consumption of stores, in direct contradiction of any belief in a beneficial effect on the colony. Colonies continuously heated throughout the winter were a little better than the controls in both respects, but their advantage was not significant, whereas there was little doubt of the adverse effects of the other heating treatments. Colonies with young queens consumed rather less stores than those with old queens.

Frequent temperature observations on twenty-five of the hives indicated that the introduction of heating units into the hive was successful in keeping up the temperature during the coldest months, but that the difference became much less in February and March, when the temperatures of unheated hives increased considerably, presumably on account of increased brood rearing. The mean hive temperature fluctuated with the outside temperature, changing by about 0·3° C. for a 1° C. change in the screen reading, except in colonies whose heating was thermostatically controlled by the screen temperature; for these the association was almost completely absent. Increases in screen temperature appeared to cause a reduction in the activity of the bees, since the highest temperature in the colony was reduced by about 0·2° C. for a unit rise in the external temperature. The lowest temperature found in the colony was closely associated with the screen temperature, though

the influence of the latter was somewhat reduced when heating was thermostatically controlled. Little evidence was found of any difference in temperature conditions between colonies heated from mid-October onwards and colonies only heated from February onwards after the beginning of the heating on the latter. Differences in temperature between colonies with old and colonies with young queens were small, but the latter were generally a little colder.

We wish to take this opportunity of thanking Mr E. B. Wedmore, of the Electrical Research Association, for suggesting this work and designing and providing the experimental heating units, and Mr Cameron-Brown for his technical assistance with the recording apparatus. Our thanks are also due to Dr C. B. Williams and Mr P. S. Milne for their assistance and advice, and to Mr D. J. Finney of the Statistical Department for considerable assistance in the analysis and presentation of the data obtained.

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THE GREY FIELD SLUG *AGRIOLIMAX AGRESTIS* L., AND ITS ENVIRONMENT

By ROBERT CARRICK, B.Sc., Ph.D.
Department of Agricultural and Forest Zoology, University of Edinburgh
(now Department of Agricultural Zoology, University of Leeds)

(With 4 Text-figures)

Ecological studies of soil invertebrates of economic importance have been largely confined to insects, and it is uncertain how far the results obtained are applicable to other invertebrates living under the same conditions. Hence it was thought that the study of a terrestrial pulmonate mollusc relative to its environment might provide useful comparisons with other unrelated members of the soil fauna.

Previous work on the ecology of land mollusca is scanty. Atkins & Lebour (1923) investigated the occurrence of snails (*Hyalinia*, *Helicella*, *Limnaea*, etc.) in relation to the *p*H of soils and natural waters. Boycott (1934) collected the references bearing upon the habitats of the land Mollusca of Britain and classified the 102 known species according to the amount of moisture and lime present in the environment. Bachrach & Cardot (1924) studied the development of *Agriolimax agrestis* and *Limnaea stagnalis* under controlled conditions of temperature. Morris (1927) showed the effect of organic manure in producing an increase of population of land pulmonates on agricultural land, and Miles *et al.* (1931) dealt with the influence of climate, soil and cultivation upon the prevalence of *Agriolimax agrestis*, *Milax sowerbii* and *Arion subfuscus*.

In the present paper an account is given of laboratory experiments on the relation of *Agriolimax agrestis* to its physical environment. These have been correlated with conditions in the field, with a view to determining what factors are contributory to slug infestations of the potato crop, and whether cultural methods based on a knowledge of the physical requirements of the slug might be so planned as to effect a measure of control, or, at least, mitigate the damage it does.

Distribution and habitat

The investigation was carried out in the Lothians of Scotland and included the area which extends from South Queensferry in the west to Dunbar in the east and is bounded by the Firth of Forth on the north, and approximately by the 500 ft. contour on the south. The land is almost entirely arable, and the chief crop is potatoes.

Slugs were prevalent in many habitats, but attention was chiefly focused on their occurrence on cultivated land, more particularly potato fields, and on hay, oats, stubble and fallow which would be followed by potatoes. The slugs common in these situations were *Agriolimax agrestis*, *Arion circumscriptus*, *A. subfuscus*, *A. hortensis*, and *A. minimus*, only the first two being present in outbreak proportions. In so far as quantitative distribution is concerned, all except the second of these species were well represented throughout the Lothians. *A. circumscriptus*, on the other hand, was markedly local in occurrence and was present at two important centres of infestation which lay respectively south-east of South Queensferry, and south of North Berwick. It occurred gregariously on ground not covered

by dense vegetation. Its favourite sites were roots of potato plants in the open field, also stones or dead leaves on bare soil; but it seldom frequented grassy banks, and its occurrence on pasture was rare.

Determination of the numbers of slugs in various habitats was not attempted, for it is difficult to find a method of computing their numbers which is at once quick and reliable. Counts of dead slugs made by applying a copper-sulphate dust at night to infested grass and stubble during winter, prior to the planting of potatoes, and in autumn immediately after cropping, gave figures varying from 30,000 to 500,000 per acre. The species concerned was chiefly *Agriolimax agrestis*, rarely *Arion circumscriptus*, which feeds underground or in sheltered situations. It was found that when the slugs increased to 100,000 per acre they occasioned severe damage to the potato crop.

Soil factors

(*a*) *Soil reaction*

Atkins & Lebour (1923) showed that snails are most abundant in neutral or somewhat alkaline soils. Although it is not exposed, a calcareous shell of reduced dimensions is developed by the Limacidae and Arionidae, and the eggs of slugs are either coated with a thin shell of calcium carbonate or possess concretions of this substance embedded in their outer coat. Also the copious white slime exuded by *Agriolimax agrestis* on irritation carries granules of the same substance. It might, therefore, be expected that the habits of this slug would bear some relation to soil acidity.

Samples of soil were collected from forty-one potato fields, and the *p*H was determined colorimetrically by means of Clark & Lubs's buffer solutions and a comparator (Fisher, 1921). The comparison between soil *p*H, slug population and damage by slugs to tubers is shown in Fig. 1. The fact that these observations refer throughout to the same kind of habitat, namely, potato fields, in all of which cultivation was identical and conditions of food and shelter were similar, serves to validate the comparison of soil factors. Estimations of the numbers of slugs present, however, had necessarily to be made at different times under variable conditions of weather, and are therefore subject to wide error. Damage to the crop, stated in terms of the percentage of tubers affected, is a more reliable index of the severity of infestation, though here again the practice of growers of lifting the crop early in order to avoid attack made comparison difficult.

There is little correlation between soil acidity, density of the slug population and the degree of damage to the crop. In the case of the six heaviest infestations, the *p*H values of the soils ranged from 5·4 to 6·9, and in those fields in which slugs were very scarce or absent the *p*H range was 4·8–7·0. Neither of the two common field slugs appears to exercise any preference for soils of a particular *p*H, for certain infestations in which *A. agrestis* predominated occurred in soils with a *p*H value of 6·9, whilst in others *Arion circumscriptus* was the dominant species in soils with *p*H values of 6·6 and 5·8. Heavy infestations comprising both species occurred at *p*H 6·2 and 5·4. There is, however, evidence that *A. circumscriptus* is more adaptable to acid soils, but this tolerance is not well defined.

(*b*) *Soil moisture*

Slugs and their eggs possess no mechanism for accommodation to changes of humidity in their surroundings. Slugs frequent moist, shady places and retire into seclusion to avoid excessive loss of water during periods of drought. Eggs become dry and are destroyed

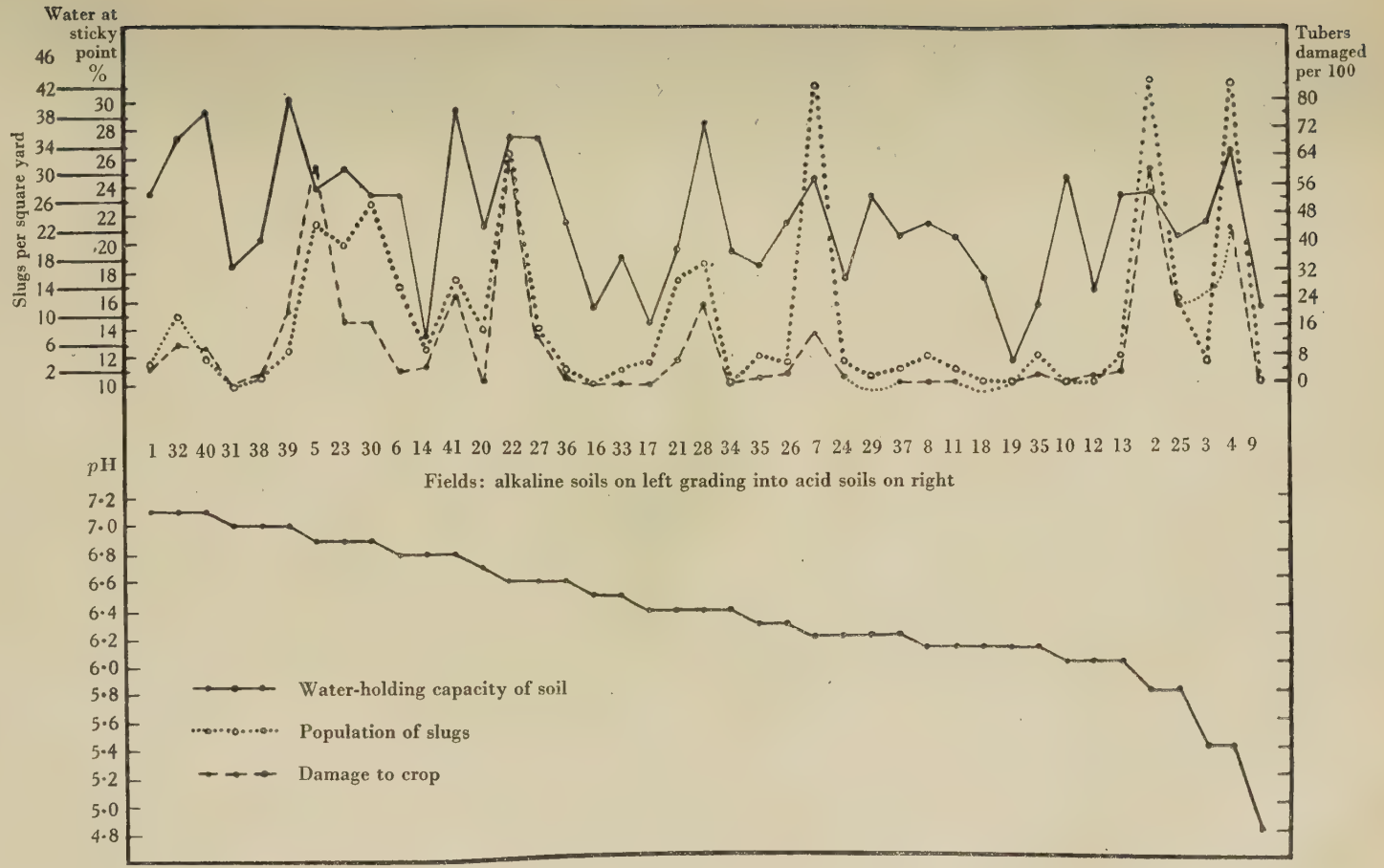

Fig. 1. Relation between soil *p*H, slug population, and damage by slugs to potato tubers.

unless contact is maintained with a moist surface. The body of *Agriolimax agrestis* has been found to consist of just over 80 % water, and the eggs contain 85 %. The obvious importance of this factor led to a study of the moisture-holding capacity of soils in the Lothians and to an investigation of the effects of the moisture content of soils upon the breeding activities of slugs.

(i) *The moisture-holding capacity of soils and the occurrence of slugs.*

The soil samples collected for *p*H determinations were also used for estimation of the moisture-holding capacity by means of the 'sticky-point' method. Fig. 1 shows that there is a close correspondence between this soil factor, the population of slugs and the damage done. The lighter and more sandy soils harboured few slugs, whilst soils with a high percentage of clay and consequent high moisture-holding capacity supported the most severe infestations. The amount of moisture which the soils held at sticky point varied from 11·82 to 30·07 %. The soils of six fields in which the populations of slugs were estimated to be greatest and damage most severe gavè an average of 25 % moisture at sticky point and a range of 23·46–27·84 %; and the nine cases in which damage was negligible and slugs very scarce showed an average of 17 % and varied from 11·82 to 24·56 %. Thus, practices which tend to increase the moisture-holding capacity of the soil at the same time render it capable of sustaining an increased population of slugs.

(ii) *Oviposition in relation to moisture content of soil.*

The eggs of *A. agrestis* become desiccated when the surface on which they rest loses moisture. Egg masses were most abundant in heavy soils and were laid usually in the first 3 in., but were not encountered in the lighter, more sandy soils, which were apt to dry out quickly on the surface during even a short period of drought.

Experiments were carried out to determine the range of soil-water content suitable for oviposition. Individuals of *A. agrestis* were kept for 2 weeks in a container and supplied with food but no soil; they were then ready to oviposit whenever a suitable medium was presented. They were placed in batches of six in closed glass vessels containing equal amounts of similar soil moistened in varying degree. The wettest soil was fully saturated, and the soil in the other four dishes was 75, 50, 25 and 10 % saturated respectively. The record of oviposition which followed is given in Table 1.

TABLE 1. *Oviposition of* Agriolimax agrestis *in soils of variable water content*

Day of oviposition	Saturation %				
	100	75	50	25	10
1	—	22 eggs on surface	26 eggs 1 in. deep	—	—
2	—	85 eggs on surface	59 eggs 1½ in. deep	32 eggs 1½ in. deep	—
3	34 eggs on surface	—	—	—	—
4	—	—	9 eggs just below surface	64 eggs 3 in. deep	—
Total no. of eggs	34	107	94	96	0

Oviposition did not occur in soil with a water content as low as 10 % saturation, despite the fact that the slugs were ripe for egg-laying. After the fourth day the water content of this soil was increased to 50 % saturation, and three egg-masses were laid within 12 hr.

Approximately the same total number of eggs was laid in each of the soils of 25, 50 and 75 % saturation, but there were differences in the time and manner of laying. At 50 and 75 % saturation, oviposition took place at the earliest opportunity, on the first night of the experiment, but at 25 % saturation it did not take place until the second night, and in fully saturated soil not until the third night. The depth at which egg masses were laid varied according to the amount of moisture present; they were placed more deeply as the soil dried on the surface.

Eggs laid in soils which were 25 and 100 % saturated did not complete their development and so failed to hatch. In the former there was insufficient moisture to keep the eggs turgid, and the embryos died during early stages of segmentation. In saturated soil the eggs were immersed, and while many embryos died at an early stage of development, a few developed into advanced embryos not far short of the condition which is attained at hatching. Other experiments designed to test the capacity of eggs to develop under water gave similar results.

The water content of soil which slugs choose as a retreat and for breeding was investigated by slanting a narrow tray containing soil with the lower end immersed in water. In this way the soil was kept flooded at the lower end, and there was a complete gradation to almost air-dry soil at the upper end. Twelve individuals of *Agriolimax agrestis* and four of *Arion circumscriptus* were left in the closed tray overnight, and in the morning four of the former species were resting on the sides of the tray close to the water-level, whilst the remaining twelve slugs, along with two egg masses of *Agriolimax agrestis*, were congregated in a narrow band of soil which proved to be 64 % saturated.

It is evident that soil must have a water content of 40–80 % saturation before oviposition followed by normal development can take place. It would appear that flooding as well as desiccation may be a physical factor which is concerned in the destruction of eggs in nature. Carrick (1938) showed that the breeding season reaches its maximum in autumn and early winter; it is determined to a large extent by the moisture content of the soil, and suitable conditions for breeding may occur at any time of year.

Binney's statement (1878) that eggs of *A. agrestis* and other slugs which had been completely desiccated for years and dried eight consecutive times in a furnace, developed and hatched normally, is too fantastic for credence. The slight resistance of the eggs to drought is a fact which has impressed itself upon all who have experimented with them.

(*c*) *Organic content*

The organic content of the soil affects slugs both directly and indirectly. Decomposing plant matter forms part of their food, and on this the newly hatched slugs largely subsist. Young slugs of a single egg mass were divided into two batches, one being placed in soil with a high organic content taken from a potato field, and the other in moist silver sand. After 6 weeks individuals of the former had increased from 3·8 mm., the size at hatching, to 6·5 mm., whilst those of the latter had grown only to 4·2 mm. Russell (1936) showed that the retention of moisture by similar soils is to a large extent dependent upon the amount of organic matter they contain. The addition of organic matter increases the water-holding capacity of the soil, making it a more favourable habitat for slugs as well as for many other members of the soil fauna. This fact was noted by Morris (1927), and Miles *et al.* (1931) stressed the importance of large applications of organic manures to the soil as a contributory factor in inducing severe infestations of slugs. The organic content of all the soils examined

in the present investigation was high, for it is the standard practice of Lothian farmers to apply to the fields 10–12 tons of farmyard manure per acre during the winter previous to planting potatoes.

Temperature

Allusion to the temperature reactions of soft-bodied invertebrates has rarely been made in the literature, and it is therefore pertinent to enquire in what degree *Agriolimax agrestis* is affected by the temperatures to which it is exposed along with other soil-frequenting invertebrates and to estimate the effects of weather upon its activities and numbers.

Laboratory experiments were undertaken to test the effects of temperature both on mature slugs and on their developing eggs. Humidity was maintained at saturation by the provision of moist filter paper, since it was found that eggs which were not in contact with a wet surface soon became desiccated. Rapid changes of temperature were avoided. Slugs which were kept at constant temperatures below 15° C. were not brought indoors. On the other hand, those which were to be subjected to temperatures of 20° C. or higher were kept in the laboratory for several days at a day temperature of approximately 18° C.; and eggs for incubation at each particular temperature were selected from those laid at or about that temperature. For constant temperatures of 15° C. and higher, electrically controlled incubators were used in the laboratory. A temperature of 10° C. was obtained by the use of an incubator kept in a cold outhouse during winter. Temperatures lower than this were provided by cold water or freezing mixtures in a large thermos jar, kept out of doors during frosty weather. The slugs or eggs were placed in vessels surrounded by the freezing mixture, and the temperature, recorded daily, did not vary more than $\pm 0{\cdot}5°$ C.

(*a*) *Control of adult activity*

Adults of *A. agrestis* were submitted to constant temperatures ranging from 35 to about 40° C. A temperature of 35° C. produced 100 % mortality after 1 hr. At 30° C., 80 % of the slugs were killed in 4 hr., although one slug withstood this temperature for 10 hr. At 25° C. activity was very slight, and all slugs recovered after 24 hr., but there was no oviposition. At 20, 15 and 10° C. activity was normal, and eggs were laid freely. At 10° C. there was only slight movement and no mortality, but eggs were not laid. There was complete inactivity at 0° C., but all the slugs submitted to this temperature recovered after 48 hr. At a slightly fluctuating temperature of about 4° C. five slugs died after 48 hr., but two slugs kept at $-5°$ C. for 2 hr. thawed out and recovered.

The most striking features of these results are the inability of *A. agrestis* to exist at a temperature of 30° C. or higher, and its ability to remain active until the temperature falls almost to freezing point. The maximum lethal temperature, which is about 35° C. in *A. agrestis*, is represented in the weevil, *Anthonomus grandis* (Hunter & Pierce, 1912) and the tick, *Ixodes ricinus* (MacLeod, 1934) by the upper limit of effective temperature. The zone of effective temperatures for *Agriolimax agrestis* extends from just above freezing point to 25° C., a range which is comparable in extent to that of insects and ticks but occurs much lower in the temperature scale.

The above results did not provide adequate information regarding the tolerance of adult slugs to freezing, but the complete recovery of two slugs after 1 hr. at $-5°$ C. indicates a very high degree of resistance. When it is remembered that the slug can seek shelter in

order to escape the rigours of weather, it is evident that the normal lower extremes of temperature in Britain would not likely be lethal to the adult slug population.

In the course of the above experiments, oviposition took place at 10–20° C., but not outside these limits. At constant temperatures of 15 and 20° C., two batches of ten slugs each laid a total of 982 and 943 eggs respectively over a period of 50 days. The higher temperature appeared to stimulate more prolific oviposition during the first 10 days. In the same period, during which the weather was frosty with an average daily maximum of 5·9° C. and an average nightly minimum of 2·0° C., a batch of ten slugs kept out of doors laid 641 eggs. Although the latter were in a condition to oviposit on the first day of the experiment, they did not do so until the sixth night. The temperature and eggs laid during the first 10 days of this experiment are given in Table 2. Under outdoor conditions the large mass of 108 eggs was laid on the first night during which the minimum temperature remained appreciably above zero, and throughout the course of the experiment the slugs postponed oviposition when the maximum temperature fell below zero.

TABLE 2. *Oviposition of* Agriolimax agrestis *at different temperatures*

Ten individuals at each temperature

	Outdoor temperatures			Experimental temperatures indoors	
Day	Max.	Min.	No. of eggs	15° C. No. of eggs	20° C. No. of eggs
1	3·3° C.	0·0° C.	—	142	113
2	3·3	−2·7	—	70	118
3	0·6	−3·3	—	39	66
4	6·7	−1·7	—	32	28
					One slug died
5	5·6	−0·6	—	19	18
6	7·7	3·3	108	51	35
7	6·7	4·4	45	5	48
					One slug died
8	4·4	2·7	—	38	56
9	3·3	−0·6	—	18	29
10	6·1	0·0	—	38	11
11 to 50	—	—	488	530	421
Totals 50			641	982	943

(*b*) *Control of development*

(i) *Incubation of the egg at constant temperatures.*

The time which elapses between oviposition and hatching, when development of the embryo takes place, varies within wide limits according to temperature. The times of incubation at constant temperatures are given in Table 3 and Fig. 2, those obtained by Bachrach & Cardot (1924) being included for comparison. Egg masses were kept in stoppered glass tubes on filter paper and submitted to constant temperatures ranging from 0 to 25° C. In order to prevent oxygen deficiency, the tubes were uncorked at intervals. Since there is a lack of uniformity of response of similar individuals to the same kind of conditions, maximum, minimum and average durations of incubation are given.

The limiting temperatures between which normal development is completed are below 5 and about 21° C. This range corresponds closely with that at which oviposition takes

place, the maximum being between 20 and 25° C. and the minimum being about 3·0° C. In Bachrach & Cardot's experiments, development took place, but hatching failed to occur at 23° C., a temperature which, in the present investigation, proved fatal to the eggs in 3 days.

TABLE 3. *Incubation of the eggs of* Agriolimax agrestis *at constant temperatures*

Temperature ...	0° C.	5° C.	10° C.	15° C.	20° C.	22° C.	23° C.	25° C.
Minimum time of incubation in days	Died in 6 days	98	53	25	15	Died in 4 days	Died in 3 days	Died in 12 hr.
Maximum do.		118	55	36	22			
Average do.		105	54	29	18			
Day degrees		525	540	435	360			
Mortality %	100	0·5	2·4	15	37	100	100	100
Bachrach & Cardot (1924):								
Temperature, ° C. ...	6	14	17·7	21	23		25	
Days	102	48	29	20·5	Failed to hatch		Failed to develop	

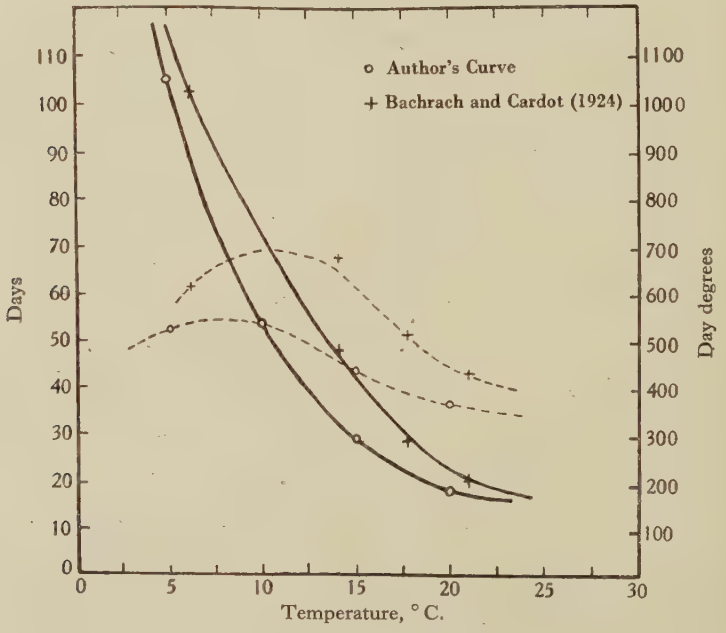

Fig. 2. Incubation of the egg of *A. agrestis* at constant temperatures.

During development there is a progressive increase in the resistance which the eggs of *Agriolimax agrestis* show to extremes of temperature. When first laid the egg is very susceptible to freezing and to temperatures higher than 21° C. But embryos which have almost completed their development can withstand a temperature of 0° C. for several days and thereafter resume development at higher temperatures, a fact which helps to explain why normal winter weather in this country effects only slight control of infestations of slugs.

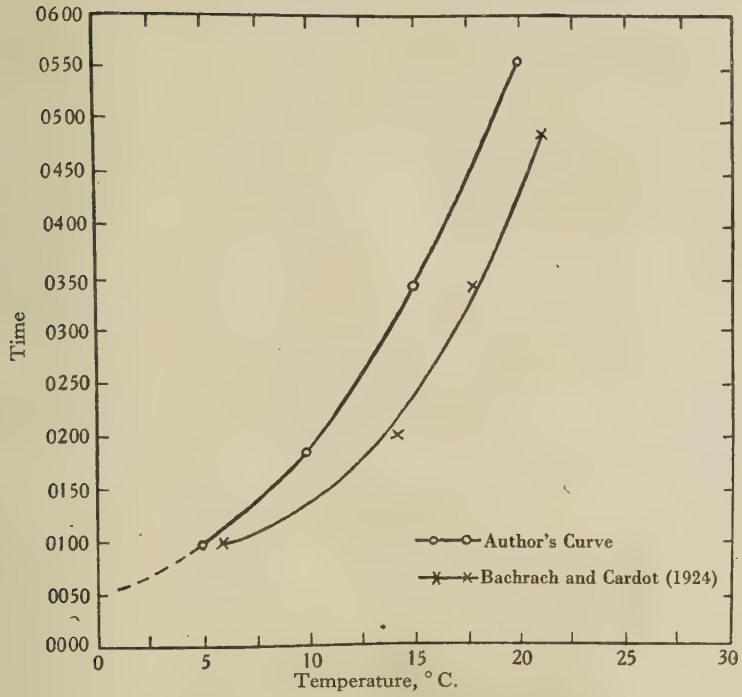

Fig. 3. Temperature-velocity curve of development for *A. agrestis*.

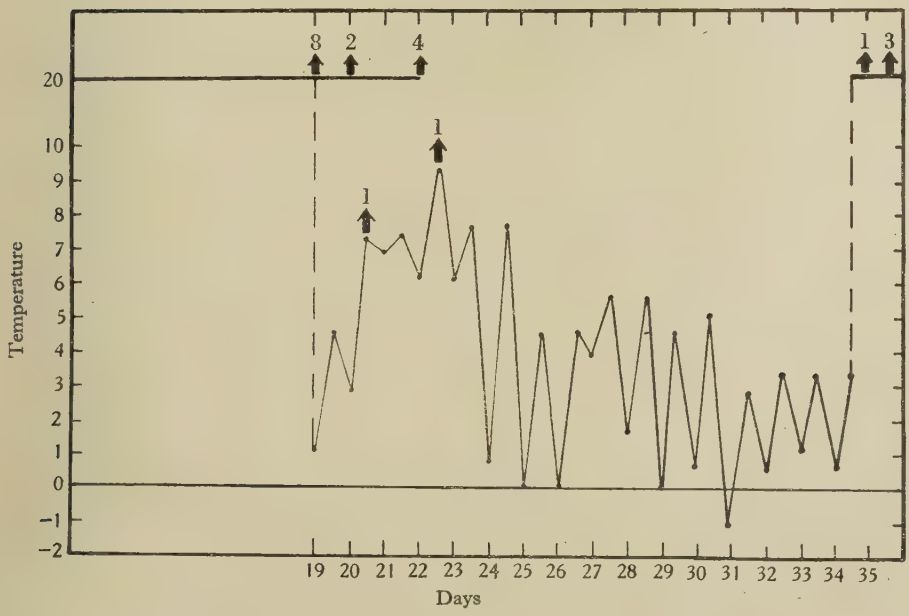

Fig. 4. Relation between hatching of eggs of *A. agrestis* and temperature.

The zones of lethal high and low temperatures at either extremity of the temperature range at which incubation occurs are confined to a few degrees, as is the case with the adult slug. In an animal which possesses no powers of temperature regulation but is directly subject to that of its environment, temperatures outside its effective range are rapidly lethal. The shortest period of development was 15 days at 20° C., but in order to determine the optimum temperature for development alone, the mortality factor has to be considered. Mortality is negligible at 5° C., but there is a rapid increase to 37 % at 20° C., a fact which is due less to the direct effects of higher temperatures on the embryo than to the encouragement of parasitic fungi, which attack the eggs.

The attempt to find a thermal constant by estimating the number of 'day degrees' has been tried, without success, in the case of many insects. The number of degrees by which the mean temperature each day throughout development exceeds that of the threshold of development is totalled, but very rarely have the figures for different temperatures been even approximately constant. In *A. agrestis* where the threshold of development can be taken as zero, there was a divergence of 180 day degrees between 360 at 20° C. and 540 at 10° C. This range is much less marked than that of the majority of insects; to take but one example, the number of day degrees required for the development of the beetle *Sitodrepa panicea* at 20° C. is 1820, a number which is practically doubled to 3638 for a fall in temperature of only 3° C.

The temperature-velocity curve of development, obtained by plotting the reciprocals of the time factor against the temperatures, shows the direct relation which a rise in temperature bears to the rate of growth. Theoretically a straight line, it has been found in the development of insects to incline less steeply towards the temperature axis at low temperatures approaching the zero of development. In the development of *Agriolimax agrestis* (Fig. 3), the curve is practically straight above 10° C., and at lower temperatures tends to turn parallel to the temperature axis, a tendency rather more pronounced in the figures of Bachrach & Cardot (1924) than in those of the present investigation. That development is thus uniform in its acceleration with increase of temperature is a further indication of the direct control exercised by this factor upon *A. agrestis*.

(ii) *Effects of temperature on hatching.*

During frosty weather in winter many eggs in the field were found to contain fully developed embryos, many of which hatched in a few hours to 2 days after being brought into the laboratory. Five fully embryonated eggs selected from an egg mass which had developed at 15° C. were frozen for 2 hr. at −2° C. and all hatched within 3 hr. of their return to 15° C., whereas the remainder of the egg mass did not commence to hatch until 2 days later. It would seem that low temperatures in the region of zero inhibit hatching, which is easily understood when it is remembered that hatching is accompanied by much muscular exertion. At the same time the change from cold to warm conditions appeared to stimulate hatching, so that relative temperatures may be more important than actual ones, no matter how near the optimum the latter may be. The effect of fluctuating temperatures on hatching, and on development both before and after hatching, merits more detailed investigation.

A mass of twenty-three eggs was incubated at a constant temperature of 20° C. until hatching commenced 19 days later. From these, six eggs were then selected, in which the

embryos were seen to be making movements of the radula preparatory to hatching. The subsequent development of these eggs took place at low temperatures out of doors during a further 16 days, when those which had not hatched were again placed in the incubator. The daily maximum and minimum outdoor temperatures were recorded, and the relation between hatching and temperature is shown in Fig. 4. None hatched on the first day, when the maximum and minimum temperatures were 4·5 and 3° C. respectively. One hatched on the second day and one on the fourth day. On the latter days the mean daily temperature was about 7° C., while from the fifth day onwards the mean daily temperature was consistently lower than 5° C., and no hatching occurred. As soon as the four unhatched eggs were transferred back to the incubator at 20° C. the embryos emerged. Between the fifth and sixteenth days the minimum temperature was frequently below 1° C., and on four occasions freezing-point was reached, without any detrimental effects to the fully developed embryos. In constant temperature experiments hatching took place at 5° C., and this can be regarded as a fairly accurate determination of the minimum hatching temperature, whilst the maximum hatching temperature is 21° C.

(iii) *Growth at various temperatures.*

Carrick (1938) showed that *Agriolimax agrestis* reaches sexual maturity, as evidenced by the deposition of eggs, when it has grown to about 2·5 cm. The time taken to attain this size is 4–6 months according to the season of the year, and, other things being equal, the controlling factor of greatest importance is temperature. Two lots of 10 slugs each, which hatched on 2 Dec., were kept, one at constant 15° C. and the other at variable temperatures outside. On 24 Apr. the average sizes were 2·90 and 1·35 cm. respectively. The number of day degrees at 15° C. was 2145, and the corresponding figure in the out-of-doors experiment, obtained by averaging daily maximum and minimum temperatures for the period, was 643. In the latter the average maximum temperature was 6·5° C. and the average minimum temperature 2·5° C. A better comparison can be made when the number of day degrees necessary for growth to the same size is taken in each case. At 15° C. a length of 1·40 cm. was attained on 25 Jan., i.e. at 55 days old, and the number of day degrees was 825 as against 643 at 2·5–6·5° C. The larger number of day degrees required for growth at the higher temperature is the converse of the results given by the developing egg, but this single result is not sufficient on which to base conclusions. It is evident, however, that temperature directly controls the rate of growth of the slug as of the egg, and it is, therefore, a vital factor in determining the time which the slug takes to reach maturity and commence reproduction in nature.

Discussion

The environmental factors of *Agriolimax agrestis*, which have been investigated individually in the laboratory, combine in nature to determine the density of the slug population. The species tolerates a wide range of soil *p*H, is dependent on the moisture content of the soil, and reacts favourably to temperatures considerably lower than those which support arthropods living in the same habitat. This susceptibility to desiccation and preference for cool temperatures explains the nocturnal activity and tendency to aestivation of the species. It remains to consider, in the light of these data, what conditions favour the development of dense populations of slugs and in what ways the depredations might be reduced.

The climate of the British Isles is well suited to the survival of *A. agrestis*, and the weather seldom produces the extreme conditions of temperature and humidity which have been shown to be lethal in the laboratory. Both summer and winter extremes, however, temporarily curtail its activity, especially egg-laying and the subsequent development of the eggs. Over the area of the slug's distribution, rainfall is nowhere deficient. The normal annual amount in the Lothians is 27 in., which is usually distributed evenly throughout the twelve months. The mean January and July temperatures are 38·5° F. (3·7° C.) and 58·5° F. (14·7° C.) respectively. Adverse natural conditions are encountered during periods of drought and heat, but are seldom intense or prolonged enough to cause a marked reduction of the population of slugs. Frost must be severe and continuous to have a pronounced lethal effect on adults, though less so on eggs, the development of which is much retarded by exposure to temperatures below 41° F. (5·0° C.).

There appear to be two ways in which the knowledge of the relation of *A. agrestis* to its physical environment may be applied in potato cultivation. Oviposition and hatching do not occur unless the temperature is maintained above zero, and development of the egg is greatly retarded at 5·0° C., while eggs recently laid soon succumb to freezing. The season of maximum oviposition is autumn and early winter, and throughout winter the further deposition and development of eggs in the field would normally decrease or cease altogether in a season of severe weather conditions. Unfortunately, the injurious effect of winter conditions on slugs is largely negatived by the application of large quantities of farmyard manure spread on grass or oat stubble. In the coldest weather, when the soil is frozen hard, egg masses laid in such manure continue to develop. The decomposing organic matter provides a plentiful food supply for the young slugs, which are further encouraged by the increased moisture-holding capacity of a heavily manured soil.

The second respect in which a knowledge of the physical requirements of the slug may be applied concerns the optimum time of cropping in relation to damage. The attack on tubers usually develops rather quickly during September, with the result that some growers harvest the crop early and so sacrifice a percentage of the yield to avoid a subsequent greater loss. In some seasons this procedure might be justified, but a comparison of weather conditions and damage year by year indicates that there are seasons distinguished by low rainfall in late summer in which this sacrifice is unwarranted. A feature of the slug population which feeds underground on tubers in September and October is the high percentage of young individuals which have apparently hatched in late summer. If a summer drought continues throughout August, reproductive activity of the slug is reduced to a minimum; on the other hand, if August is a month of high rainfall, there will develop large numbers of young slugs capable of inflicting serious damage to tubers during late September and October. The aim would thus be to lift the crop just before the slugs have had time to commence serious damage.

Summary

The physical environment of the grey field slug (*Agriolimax agrestis* L.) has been studied in the laboratory, and the results have been correlated with conditions in the field with particular reference to the occurrence of outbreaks on the potato crop in the Lothians of Scotland. The slug tolerates a wide range of soil *p*H. Soils of high water-holding capacity sustain the densest populations of slugs, and they must be 40–80 % saturated before

oviposition followed by development of the eggs can occur. The maximum lethal temperature is about 35° C.; the zone of effective temperatures extends from just above freezing-point to 25° C., and there is a high degree of resistance to freezing. Oviposition occurs anywhere between 3 and 20° C. Time of development of the egg varies from 105 days at 5° C. to 18 days at 20° C., and mortality of the egg increases from zero at 5° C. to 37 % at 20° C. Hatching of the egg is inhibited below 5° C. Temperature exercises a direct controlling effect on development. Normal extremes of weather in Britain are not usually lethal to *A. agrestis*, but they do serve to inhibit adult activity and reproduction. In winter, manuring of the fields tends to counteract the lethal effect of adverse weather, and rainfall in late summer is important in determining the extent of damage by slugs to potato tubers during autumn.

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BAITING SLUGS USING METALDEHYDE MIXED WITH VARIOUS SUBSTANCES

By H. F. BARNES and J. W. WEIL
Rothamsted Experimental Station, Harpenden

(With 6 Text-figures)

The fact that metaldehyde mixed with bran will cause numerous slugs to remain on the surface of the ground, where they die, in the neighbourhood of heaps of this mixture is generally known. But there is little reliable information available concerning its exact mode of action on slugs, its efficiency compared with other baits (Cameron, 1939; Lange & MacLeod, 1941), and its efficacy in clearing an area of slugs.

The purpose of this paper is to review briefly what is known concerning the use of metaldehyde alone or mixed with other substances as a slug bait, to give the results of some experiments using metaldehyde mixed with various substances as possible alternatives for bran in the mixture and to deal with the efficiency of this type of baiting.

Review of literature

The compound metaldehyde is the chief constituent of a solid fuel, marketed under the trade name 'Meta', a product of Swiss origin and also as 'Metaldehyde' manufactured in Britain. The exact nature of the discovery that it is fatal to slugs is veiled in mystery, but it is clear that this fact was known in South Africa in 1934 (Gimingham, 1940). The first mention of meta in this connexion in the English press was by Hadden (1936) who recommended its use mixed with bran. Further references quickly followed in the scientific, horticultural and daily press.

Gimingham & Newton (1937) gave figures to show that the compound metaldehyde was probably just as efficient in catching large numbers of slugs when it was mixed with bran as was meta, but that metaldehyde was at that time more expensive. Jary & Austin (1937) showed that meta alone was attractive to slugs and also when mixed with sand or soil, but unfortunately did not give any figures comparing the numbers caught by meta mixed with bran and those caught by meta alone, meta with sand or meta with soil, although they appreciated the well-known fact that bran by itself has an attraction for slugs. Nicholson (1937) had previously stated that he did not know why bran was used except as a spreader. Newton (1937), however, stated that bran mixed with meta was better than meta alone, both because of the mechanical difficulty in using meta alone and, what was just as important, because bran itself is attractive. This fact has tended to become overlooked. Oldcorn (1938) stated that he always used meta by itself, and that meta powder mixed with equal proportions of flowers of sulphur gave exactly the same results. Esslemont (1938) found undiluted meta too expensive and meta with sand gave equally good results, but was also more costly than using meta with bran. Pethybridge (1938) stated that ground lime as a diluent for meta gave satisfactory results. Cameron (1939) stated that meta could be used as a powder alone, but that its lightness made distribution difficult, or with an inert diluent, e.g. sand. Jary (1939) went so far as to state that there was no evidence that a bran mixture was more effective than an inorganic diluent. It was suggested (Anon. 1940) that grass clippings should be used in war time as a substitute for bran. This was quickly followed by the suggestion by Pratt (1940) that dried lawn clippings or sand should be used for the same purpose. He also stated that in the U.S.A. ground oatmeal was used. Nash (1940) reported that lime or even sulphur was quite effective. Lastly, Somerset (1941*a*) stated that sawdust should be used and later (1941*b*)

defended this statement by saying that slugs do not eat the substance with which the meta is mixed, that meta kills on contact, that the carrier is unimportant, and that it was a waste to use good bran when sawdust will answer the same purpose.

Many of the statements referred to above are characterized by the almost complete lack of figures substantiating their claims and of any comparison of the kills obtained by these alternative substances.

META OR METALDEHYDE WITH VARIOUS SUBSTANCES AS POSSIBLE SUBSTITUTES FOR BRAN

All the present experiments, of which brief reports have already been published (Barnes & Weil, 1940, 1941) in the horticultural press, have been carried out in the garden of Old Orchard, Moreton End Lane, Harpenden, during 1940 and 1941. Forty-four substances have been tested as possible substitutes, and their kills, when mixed with metaldehyde, compared with those obtained by metaldehyde-bran baits. Twenty experiments have been conducted involving the capture of 13,362 slugs of various species and the use of 367 heaps of bait exposed on 60 nights. The Swiss-made meta was used in all the experiments (I–XV) carried out in 1940, while the British-made metaldehyde took its place in the experiments done in 1941 (XVI–XX).

Since slugs are nocturnal in their habits, the baits were put out in the early evening, and during their first night's exposure the slugs assembled round them were counted and removed just before midnight and again about 8 a.m. Sometimes, however, the count during the night was omitted. The slugs were also counted and removed each subsequent morning for a period of 3–9 days. In one experiment (XIXA) the slugs were counted and removed hourly during the first night. In each experiment bran mixed with powdered meta or metaldehyde was used as a standard of comparison. With two exceptions, viz. wet liquorice marc and spent hops, all baits, including the bran, were put out dry; they soon became damp by absorption or precipitation. The quantity (about 170 c.c.) of each substance used was enough to fill a 2 oz. flat tobacco tin ($4\frac{1}{2} \times 3 \times \frac{4}{5}$ in.). With bran, this volume weighed 35 g. To this was added a bar (2 tablets; 5 g.) of powdered meta or metaldehyde except when proprietary mixtures already containing aldehyde and when bran without metaldehyde were being tested. This quantity was usually divided into fifths (in two experiments, I and X, into sevenths and once, Exp. VIII, into fourths) to enable replication. In this way a comparison was obtained between the number of slugs caught by bran and each of the other substances within any one experiment.

Jary & Austin (1937) stated that the majority of slugs were attracted during the first two or three nights: this was quickly confirmed. Most of the slugs were caught during the first night's exposure of the baits, and usually after the fourth or fifth night the numbers caught were insignificant, staying more or less constant for so long as the baits remained in working order. The numbers of slugs caught per night in seven experiments are shown in Fig. 1. Out of a total of 4684 slugs caught in the seven days, 2577 or 55 % were caught during the first night, 836 or 18 % on the second, and 485 or 10 % on the third. This represents 83 % on the seven night's captures.

Fig. 2 shows the numbers of slugs (Exp. XIXA) attracted per hour throughout one night to heaps of three baits (bran only, bran mixed with powdered metaldehyde and powdered metaldehyde alone). From Table 1 it will also be seen that the proportion caught before and after midnight seems to vary with the season, fewer before midnight in the spring than in the early autumn. The percentage caught before midnight during the period 28 Aug.–7 Sept. is significantly greater than the percentage caught during the earlier period.

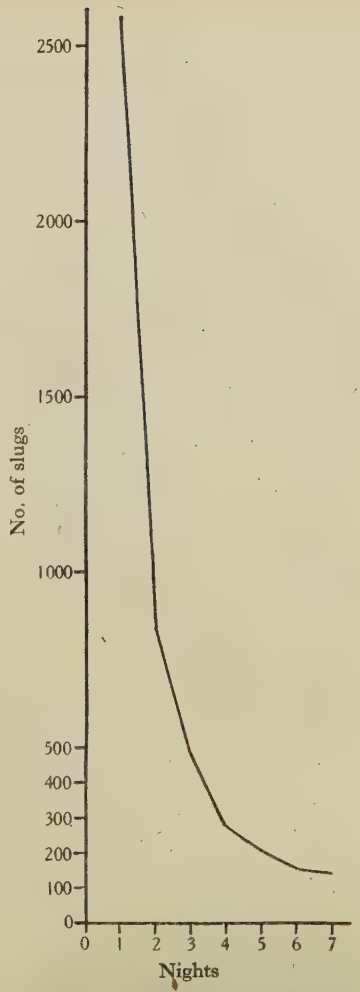

Fig. 1. Number of slugs caught per night in seven experiments.

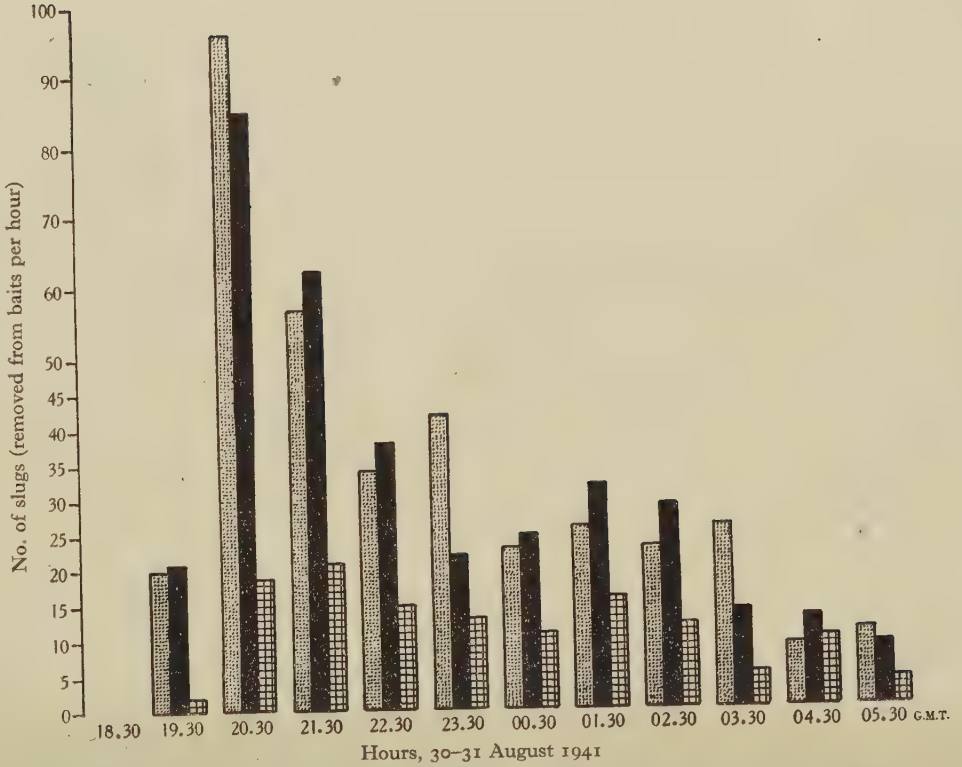

Fig. 2. Number of slugs removed hourly from baits of (*a*) bran without metaldehyde ▨; (*b*) bran mixed with metaldehyde ■; (*c*) powdered metaldehyde alone ▦.

The reason for this is not definitely known, but it is possible that different species of slugs have different times of nocturnal activity, and that different species predominate in the garden at different seasons of the year. For example, a sample of 195 slugs collected at random in ½ hr. one night during September comprised 155 *Agriolimax agrestis*, 3 *Milax sowerbii*, 12 *Milax gagates*, 24 *Arion subfuscus* and 1 *Arion ater*. We are indebted to Miss Barbara H. Wright for these identifications.

The number of slugs caught in the first four nights' exposure of the substances tested are shown in diagrammatic form in Fig. 3. Where the number of slugs caught is significantly higher or lower than that caught by bran (and metaldehyde) in the same experiment, the fact is indicated by an asterisk at the top of the column, two such signs representing a 1 % significance, and one such sign a 5 % significance.

One striking fact is that after nineteen experiments, instead of getting smaller numbers of slugs, the highest number ever reached in a single experiment was obtained. The twentieth experiment was, however, made in September, while the first was carried out in May in the previous year.

TABLE 1. *Slugs caught before and after midnight*

Period of experiment	No. of exps.	No. of slugs caught on first night	% caught	
			Before midnight	After midnight
29 Apr.–8 May	6	2612	38	62
20 July–1 Aug.	4	1177	41	59
28–31 Aug.	2	1183	58	42
6–7 Sept.	1	1284	77	23

Different baits may differ in their attraction to the various species of slugs, and if any species was predominating the results would be weighted by the degree of attraction to this species of the baits used. Thus a spring species might be expected to be attracted more to baits containing something akin to their natural food, i.e. plants such as lettuce which are abundant in the spring, than to other substances which are more readily available in the autumn, e.g. cabbage and potato leaves or tubers. As has already been suggested, different species may predominate at different seasons of the year.

Table 2 gives the percentage differences from bran of the number of slugs captured when using various substances instead of bran, metaldehyde being added in every case except where metaldehyde alone was used. The figures used are the totals obtained for all nights of the experiments. The substances are arranged in two columns, feeding stuffs and non-feeding stuffs, and in groups according to their use or origin. Two asterisks indicate a 1 % significant difference. It will be seen that if the catch of slugs by the bran bait is taken as 100, then, considered in groups, animal foods capture 127, human foods 109, commercial by-products of feeding value 74, tea and coffee wastes 71, farm and garden produce 46, commercial by-products of no feeding value 40, and miscellaneous substances (metaldehyde alone and with inert substances) catch 22. These differences all show a 1 % significance except in the case of human foods.

There is a remarkable consistency of results obtained within the groups other than those of human and animal foods, the only exception being dried and powdered grass cuttings (see footnote to Table 2). The variation within the groups of human and animal foods is,

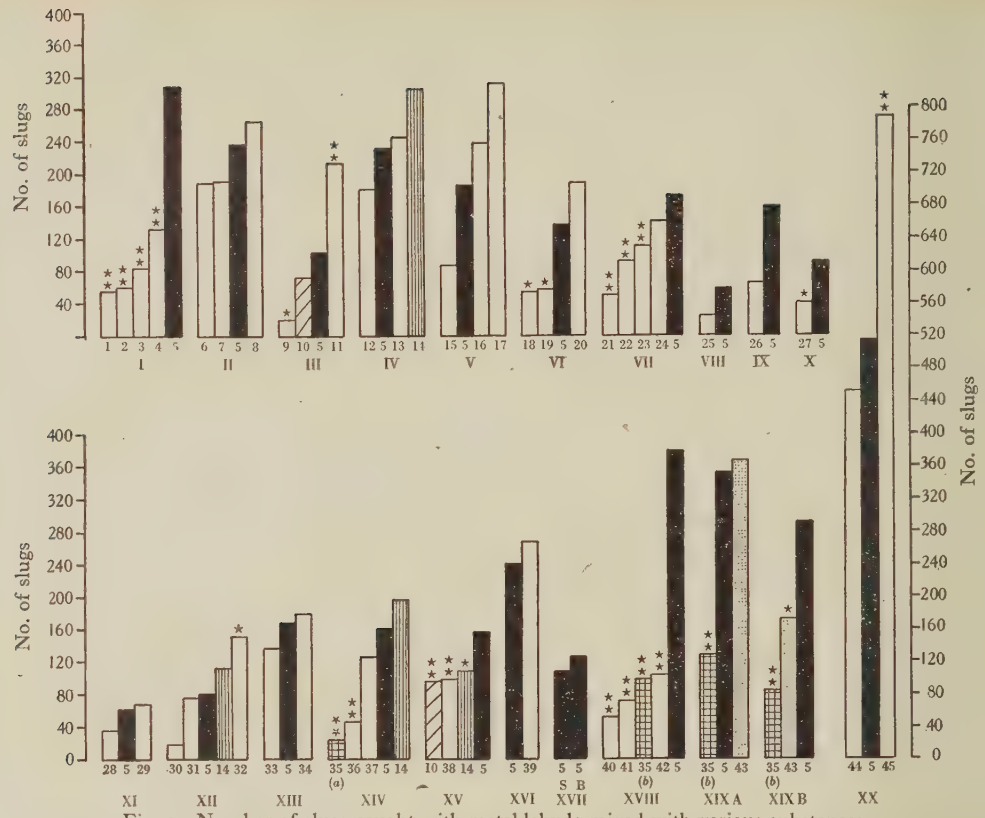

Fig. 3. Number of slugs caught with metaldehyde mixed with various substances.

Each histogram represents the number of slugs caught during the first four nights in Exps. I–XX, except in Exp. VII in which they are the totals of the first five nights, in Exp. XIXA in which they are the totals of the slugs removed each hour during the 1st night of the experiment, and in Exp. XIXB in which they are the totals of the slugs caught on the 2nd, 3rd, 4th, and 5th nights of the experiment.

Each substance indicated by an arabic number was mixed with 'meta' or 'metaldehyde', the Swiss 'meta' being used in the first fifteen experiments and the British 'metaldehyde' being used in the last five experiments. The exception is 43 when bran was tested without the addition of any metaldehyde.

In Exp. XVII the Swiss-made 'meta' (S) was compared with British-made 'metaldehyde' (B) both mixed with bran.

Where the number of slugs caught is significantly higher or lower than that caught by bran (and metaldehyde) in the same experiment, the fact is indicated by asterisks at the top of the columns, two such signs representing a 1 % significance and one such sign a 5 % significance.

1. Soil
2. Rhubarb leaves
3. Cabbage leaves
4. Lettuce leaves
5. Bran ■
6. Quaker oats
7. Ground rice
8. Flour
9. Newspaper
10. Tea leaves (used) ▨
11. Middlings
12. Chicken meal
13. Brown biscuit meal
14. Yellow maize meal ▥
15. Proprietary C
16. Proprietary B
17. Proprietary A
18. Oats (whole grain)
19. Wheat (whole grain)
20. Cattle cake
21. Sawdust
22. Proprietary D
23. Molassed sugar-beet dust
24. Sugar-beet pulp
25. Lawn grass cuttings (fresh)
26. Derris residue
27. Liquorice marc (wet)
28. Scammony marc (dried)
29. Cotton cake
30. Pyrethrum marc (dried)
31. Flaked yellow maize
32. Special meal
33. Ground-nut cake
34. Linseed cake

35*a*. 'Meta' whole bar } ▦
35*b*. 'Metaldehyde' powdered }

36. Spent hops
37. Coffee-bean offal
38. Coffee grounds
39. Castor meal
40. Flowers of sulphur
41. Chalk (calcium carbonate)
42. Sand
43. Bran without metaldehyde ▦
44. Lawn grass cuttings (dried and powdered)
45. Brown bread crumbs (dried and powdered)

however, wide, but this may be ascribed to the great differences in the characteristics of the foods, e.g. between brown bread and linseed cake. In spite of these differences no single food is significantly less effective than bran and it appears that, on the average, feeding stuffs are significantly more effective than bran.

TABLE 2. *Percentage differences from bran of slugs captured when using possible substitutes*

Feeding stuffs	
Human foods:	
Brown bread crumbs (dried and powdered)	$+53 \pm 13{\cdot}3$
Flour	$+18 \pm 20{\cdot}0$
Ground rice	$-15 \pm 20{\cdot}0$
Quaker oats	$-18 \pm 20{\cdot}0$
Mean	$+9{\cdot}5 \pm 9{\cdot}3$
Animal foods (concentrates):	
Cattle cake	$+42 \pm 18{\cdot}1$
Cotton cake	$+31 \pm 25{\cdot}8$
Ground-nut cake	$+12 \pm 22{\cdot}8$
Linseed cake	$-15 \pm 22{\cdot}8$
Middlings	$+84 \pm 24{\cdot}2$
Special meal	$+89 \pm 36{\cdot}1$
Yellow maize meal	$+50 \pm 36{\cdot}1$ $+35 \pm 22{\cdot}5$ $+24 \pm 13{\cdot}3$
Castor meal	$+16 \pm 23{\cdot}1$
Brown biscuit meal	$+6 \pm 22{\cdot}5$
Flaked maize	$-2 \pm 36{\cdot}1$
Chicken meal	$-17 \pm 22{\cdot}5$
Mean	$+27{\cdot}3 \pm 7{\cdot}2$**
Farm and garden produce:	
Lawn grass cuttings† (dried and powdered)	$-12 \pm 13{\cdot}3$
Lawn grass cuttings (fresh)	$-61 \pm 26{\cdot}1$
Cabbage leaves	$-57 \pm 17{\cdot}1$
Lettuce leaves	$-73 \pm 17{\cdot}1$
Oats (whole grain)	$-62 \pm 18{\cdot}1$
Wheat (whole grain)	$-61 \pm 18{\cdot}1$
Mean	$-54{\cdot}3 \pm 7{\cdot}6$**
Commercial by-products:	
Sugar-beet pulp	$-16 \pm 10{\cdot}7$
Molassed sugar-beet dust	$-36 \pm 10{\cdot}7$
Mean	$-26 \pm 7{\cdot}6$**

Non-feeding stuffs	
Commercial by-products:	
Scammony marc dried	$-36 \pm 25{\cdot}8$
Derris residue	$-60 \pm 24{\cdot}4$
Pyrethrum marc dried	$-71 \pm 36{\cdot}1$
Liquorice marc wet	$-55 \pm 22{\cdot}2$
Spent hops	$-72 \pm 13{\cdot}3$
Sawdust	$-67 \pm 10{\cdot}7$
Mean	$-60{\cdot}1 \pm 9{\cdot}6$**
Tea and coffee wastes:	
Tea leaves	$-28 \pm 24{\cdot}2$ $-36 \pm 9{\cdot}4$
Coffee grounds	$-30 \pm 9{\cdot}4$
Coffee-bean offal	$-22 \pm 13{\cdot}3$
Mean	$-29 \pm 7{\cdot}7$**
Miscellaneous:	
Meta bar	$-84 \pm 13{\cdot}3$
Metaldehyde powdered	$-72 \pm 13{\cdot}1$ $-71 \pm 12{\cdot}8$
Sand	$-73 \pm 13{\cdot}1$
Soil	$-81 \pm 17{\cdot}1$
Flowers of sulphur	$-85 \pm 13{\cdot}1$
Chalk	$-81 \pm 13{\cdot}1$
Newspaper	$-77 \pm 24{\cdot}2$
Rhubarb leaves	$-80 \pm 17{\cdot}1$
Mean	$-78{\cdot}2 \pm 5{\cdot}2$**

† This has been placed in the farm and garden produce group, although it would be included in the animal food group if produced on a commercial scale.

From these data it is apparent that different numbers of slugs are attracted to different baits. Larger numbers of slugs are caught by metaldehyde baits containing material on which the slugs can feed than by similar baits containing substances on which they presumably do not feed. Nutritive feeding stuffs, such as cattle cakes and meals, taken as a group appear to be more attractive than roughage feeding stuffs, such as bran. Bran when used without metaldehyde will attract large numbers of slugs and in addition cause considerable numbers of them to remain exposed on the surface of the ground the following day, this resulting in their death by birds or exposure to desiccation. Such numbers are

significantly lower than those caught by bran with the addition of metaldehyde but significantly higher than those caught by powdered metaldehyde alone. Metaldehyde alone will catch quite a lot of slugs but much fewer than when it is mixed with a feeding stuff (Table 3).

TABLE 3. *No. of slugs caught by bran mixed with metaldehyde, bran alone and metaldehyde alone* (*Exp.* XIXB)

	No. of slugs	Difference ±40·0
Bran with metaldehyde	293	—
Bran alone	174	−119
Metaldehyde alone	84	−209

The feeding stuffs in the baits are the main source of attraction, while the powdered metaldehyde is the main cause of the slugs remaining on the surface round the baits the following day. But the numbers of slugs caught in experiments using metaldehyde alone are too high to be accounted for solely by chance approach to the baits. The numbers of slugs caught by metaldehyde alone resemble the numbers caught when using metaldehyde mixed with inert substances. In other words, metaldehyde does exert some attraction, and the tests with baits containing metaldehyde with inert substances were for all practical purposes further trials on the effect of metaldehyde alone. This is shown by the figures of mean differences, viz. $-75{\cdot}7 \pm 7{\cdot}5$ for the three experiments using metaldehyde alone and $-74{\cdot}0 \pm 6{\cdot}5$ for the six experiments using metaldehyde with inert substances, namely, sand, soil, flowers of sulphur, calcium carbonate, newspaper and sawdust. It is clear that in a mixed slug population the only use for such inert substances is as a carrier or spreader of the metaldehyde.

Efficiency of metaldehyde baits

This depends (*a*) on the durability of the baits, (*b*) their attractiveness, and (*c*) their ability to cause the death of the slugs after they have visited the heaps.

Durability

It is obvious that the weather, particularly rain, the liability of the baits to turn sour or go mouldy and the relative attractiveness of the baits to other animals, will all affect the durability as much or more than the duration of the inherent attractiveness of the baits themselves. Some observations made on these points may be useful.

Metaldehyde is almost insoluble in water and so rain has little effect on it except to wash it away. Some workers have suggested covering the heaps of bait as a safeguard against the weather as well as against the possibility of poisoning of birds or domestic animals.[1] A disadvantage of this is that bran remaining continuously damp becomes mouldy sooner than when allowed to dry up during the daytime in fine intervals and other substances would probably be similarly affected. Flour and calcium carbonate set into a hard paste after rain and in this way were proof against high winds which tended to scatter some of the other baits when they became dry on fine days. On the other hand, in dry weather slugs tend to become inactive and then rain increases the numbers caught by the baits (Fig. 4).

[1] It was the custom of the Swiss manufacturers of meta to add a distasteful substance to the bars. Special care should always be taken to prevent dogs having access to baits which would be attractive to them. Broadcasting the bait instead of putting it out in heaps would practically eliminate any risk of birds or domestic animals obtaining a fatal dose.

Powdered bread crumbs, sugar-beet pulp, and molassed sugar-beet dust moulded after a few days; middlings, bran and special meal after the first week; and yellow maize meal and biscuit meal after about 12 days. A direct test of the durability of yellow maize meal was

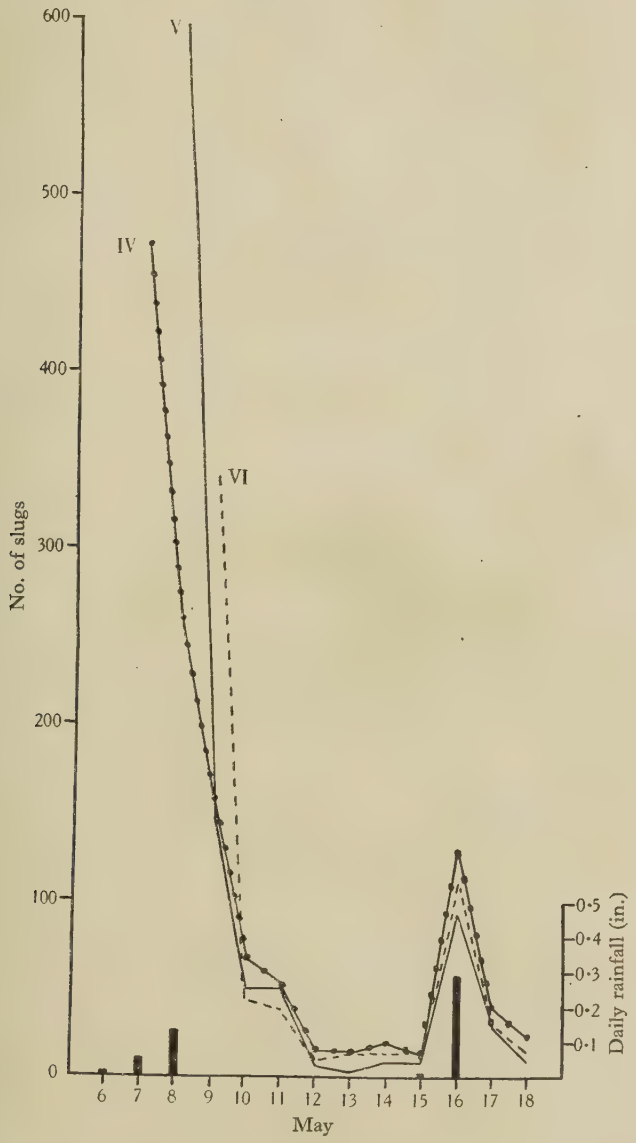

Fig. 4. Effect of rain on numbers of slugs caught (Exps. IV, V and VI)

made. In Exp. XII the number of slugs captured by yellow maize meal and meta was not significantly different from that caught by bran and meta. After these baits had been exposed for six nights, Exp. XIV was started in new positions. New bran baits were used

but the yellow maize meal baits used in Exp. XII were transferred with as little disturbance as possible. In the six nights this experiment was conducted there was still no significant difference between the fresh bran and the six-nights-old yellow maize meal. In Exp. XV new sites were chosen and the yellow maize meal baits which were by this time becoming mouldy were used once more and compared with fresh bran baits. This time the yellow maize meal baits showed a 5 % significant reduction in the number of slugs caught. Fig. 5 shows the percentage differences, from the three sets of fresh bran baits, of the nightly captures on the yellow maize meal baits which were used for 18 consecutive nights.

However, such a very large proportion of the catch is obtained in the first 3 days (see Fig. 2) that it is not really important if the feeding stuff bait goes mouldy after several days when these baits are being used to reduce rapidly a large population. It would be a

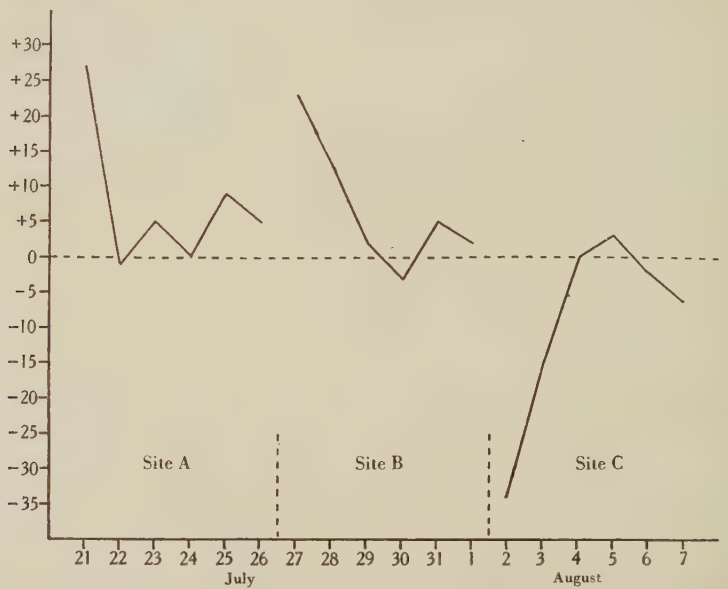

Fig. 5. Differences in numbers of slugs caught by yellow maize meal baits exposed continuously for 18 days and bran baits which were renewed after each 6 days' exposure. All baits were in new sites each 6 days.

disadvantage if such baits were used to prevent a population from developing to dangerous proportions. For this reason inert substances are recommended later in this paper for use as preventive baits and feeding stuffs as depopulating baits.

The speed at which the moulds grow must depend largely on the atmospheric moisture. Any step taken to preserve a high humidity, such as covering, would aid their growth, so advantage should be taken of any dryness during the day, when slugs are not active, by leaving the baits freely exposed to the wind and the sun. In spite of this some substances will become mouldy and unpalatable before others.

Although metaldehyde itself seems to be attractive and poisonous to a marked extent only to slugs and snails, some of the baits used are attractive and eaten by other animals and insects. For this reason some substances remain available as effective slug baits for a shorter period than others. For example, quaker oats and ground rice were very attractive to a

hedgehog which if allowed to remain might soon have spoilt any efficacy these substances had as an attractant for slugs. Fortunately, however, the meta did not agree with the hedgehog which became ill and, after what appeared to be an hour or so of bad indigestion, moved away. Earthworms proved very partial to the lettuce, cabbage and rhubarb leaves used in the first experiment and also to the fresh grass cuttings in Exp. VIII. Mice were strongly attracted to the whole oats and wheat grains used in Exp. VI apparently with no ill effect to themselves. Flour and ground rice proved attractive to woodlice and millipedes which arrived each night to feed, but none was found dead in the vicinity. Bran is also known to be attractive to leatherjackets but metaldehyde-bran baits are not fatal to them.

Attractiveness

A bait may or may not be as attractive later as on the first night it is exposed. Besides this, different baits probably have different ranges of attraction. No definite information can be obtained in field work on these points until a satisfactory method has been found of marking slugs. In addition, if this were possible, the population could be estimated and the efficiency of any particular bait measured directly. One experiment in marking slugs by threading them with coloured cottons was made, but no slugs were recovered after the first night.

Ability to cause the death of slugs visiting the baits

Metaldehyde is known to bring about the death of slugs by causing them to remain on the surface of the ground, but it is not known what proportion of the slugs which visit the baits remain on the surface. It is proposed to get evidence on this point by observing hourly, but not removing, the number of slugs visiting the baits, and then counting the number remaining on the soil surface in the vicinity of the baits the next morning.

Meanwhile it may be assumed that 100 % of the slugs found on the surface out of doors the following day will either die by desiccation or by being eaten by birds, hedgehogs, beetles and other animals. Neither of these results would necessarily occur to the same extent under cloches or in glasshouses. In addition, some of the slugs that are still sufficiently active to take cover by daybreak may die.

The exact manner in which the compound metaldehyde acts on the slugs is not known. But it has been shown that both contact with metaldehyde and feeding on metaldehyde and substances containing metaldehyde will cause slugs to 'become comatose and capable only of restricted movement. They are thus unable to reach shelter when daylight appears and death follows more or less rapidly according to the amount of desiccation to which they are then subjected' (Newton, 1937). A further well-known observation may be added. Contact with and the eating of metaldehyde seems to induce an excessive secretion of slime and this may result in the slugs being unable to regain shelter both because of bodily weakness and mechanical difficulties.

Discussion

The question arises as to whether the use of feeding stuffs in the baits is economical or not. It is obvious that large numbers of slugs can be caught by the use of a small amount of bran, and that such a number of slugs can do a vast amount of damage. Thus, in Exps. I–XX, 1 lb. 9 oz. of bran was used and at least 4616 slugs were destroyed. In addition twenty bars of meta or metaldehyde were used. Thus the total expenditure was 2*s*. It is known that this

is roughly $\times 5$ the quantity of metaldehyde which Gimingham & Newton (1937) and Esslemont (1938) proved to be efficient in killing slugs. This excess of metaldehyde was used on purpose to minimize the risk of having a non-effective killing agent. Since the cost of the metaldehyde was about four-fifths of the total cost of metaldehyde-bran baits, the cost can safely be reduced considerably by reducing the quantity of metaldehyde used.

If, however, the proportion of metaldehyde is reduced, difficulties of obtaining a satisfactory and even mixture of the metaldehyde and diluent become relatively much greater. Even when one bar of metaldehyde was mixed with 35 g. of bran in the experiments, it was found that shaking the bait quickly caused the powdered metaldehyde to become separated from the bran. From this it is apparent that the mere cartage of such a mixture across a field would tend seriously to separate out the bran from the metaldehyde and so cause parts of the baits to lose their efficiency. Pratt (1940) pointed out that ground oatmeal mixtures do not disintegrate in the same manner as bran mixtures. If the bran were damped it would reduce the risk of the metaldehyde separating from the bran, but, on the other hand, it would increase the chances of getting an initial uneven mixing. If, instead of a flaky substance such as bran, a powdery substance such as yellow maize meal or brown bread crumbs or biscuit dust were used, it would be much simpler to retain an even mixing. Sand would also have this advantage. But it has been shown that metaldehyde-sand baits do not catch such large numbers of slugs as metaldehyde mixed with a feeding stuff.

It might be argued that since it has been shown that metaldehyde itself does attract slugs, a slug population could be reduced by metaldehyde diluted with an inert substance. This is true, but the speed of capture would probably be slower. The curves of nightly catches of slugs for inert substances fall away less rapidly than those for feeding stuffs, chiefly on account of the lower initial catches. But the curves for inert and less nutritive substances do not, even after several days, rise above those for feeding stuffs, so that the total catch is influenced largely by the results of the first three or four nights and increasing the number of nights would not change the results. Fig. 6 shows the curves for the nightly captures of (*A*) bran mixed with metaldehyde and (*B*) of inert or miscellaneous substances as a group.

The percentage caught on the first three nights in this set of experiments is 79 as compared with 83 in seven other experiments (see p. 57 and Fig. 1).

Undoubtedly, if sufficient numbers of baits were put out, the possible first advantage of using an attractive diluent with a possible wider range of attraction than metaldehyde with an inert diluent might be overcome to some extent. The speed of reducing a slug population is, however, of great importance. Moreover, if the number of metaldehyde plus inert substance baits can be increased so as to increase the range of attraction and speed of depopulating an area, equally so can the number of metaldehyde plus feeding stuff baits be increased with the same results. The speed of depopulating an area of slugs by using baits is so important that it will be further investigated.

In the present state of knowledge, it would appear better to use a small amount of a feeding stuff, for preference, perhaps, a roughage one rather than a concentrate, unless roughage is scarce, in order to destroy quickly large numbers of slugs. The amount of crops thus saved would far outweigh in value as feeding stuffs the amount expended in their preservation.

It has been shown earlier in this paper that the majority of the slugs are caught during the first few nights. It can be presumed that, providing enough baits are used, the slug popula-

tion in an area would be reduced by a single application. Also it has been shown that household refuse such as tea leaves and coffee grounds with metaldehyde, although attracting significantly fewer slugs than bran, do catch appreciably more than metaldehyde with an inert diluent. For these reasons, it may be suggested that in cases where slugs have become a pest, a first treatment with a metaldehyde plus feeding stuff bait should be used to decrease the population as quickly as possible. Then a second application of baits containing metaldehyde plus an inert diluent such as soil or sand would be suitable in order to prevent the slug population attaining dangerous size again. In small areas it might be more advantageous to follow the first baiting with a second baiting by tea leaves or coffee grounds with metaldehyde in order to complete as quickly as possible the destruction of the initial population. Then, finally, as a long period preventive, metaldehyde plus soil might be applied which should last for several months, especially under cloches and in glasshouses which are protected from the heavy rains which could wash the metaldehyde down in the soil.

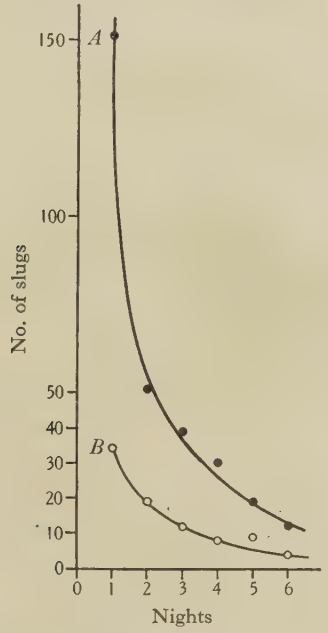

Fig. 6. Curves for nightly captures of slugs by *A*, metaldehyde mixed with bran and *B*, metaldehyde mixed with inert or miscellaneous substances.

Summary

The literature concerning mixtures of other substances with metaldehyde for use as slug baits has been briefly reviewed. An account has been given of the comparison of the numbers caught by forty-four substances and by bran in metaldehyde baits. It is shown that baits containing feeding stuffs are undoubtedly better than baits of metaldehyde alone or metaldehyde mixed with non-feeding stuffs, such as soil, sand, etc. Nutritive feeding stuffs, such as cakes and meals, are better as a group than roughage feeding stuffs, such as bran. The efficiency of metaldehyde baits is discussed. The range of effectiveness of the baits, the

proportion of slugs visiting the baits dying, the speed of annihilating a slug population, the most economical strength of metaldehyde to use as well as the mixing of the baits and their distribution are points which need further investigation. Meanwhile, it is suggested that it is worth while using small quantities of a feeding stuff in order to save much larger quantities of crops which are themselves feeding stuffs. A large slug population can be greatly reduced by a single application of metaldehyde plus a feeding stuff and can be subsequently prevented, by the use of metaldehyde and an inert or non-feeding stuff diluent, from reaching undue proportions again.

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ADDENDUM

See p. 59. Since going to press, further sampling has shown that four other species, namely *Limax maximus*, *Arion circumscriptus*, *Aribn hortensis* and *Arion intermedius*, are also present in the garden.

THE PROBLEM OF THE EVALUATION OF ROTENONE-CONTAINING PLANTS

VI. THE TOXICITY OF *l*-ELLIPTONE AND OF POISONS APPLIED JOINTLY, WITH FURTHER OBSERVATIONS ON THE ROTENONE EQUIVALENT METHOD OF ASSESSING THE TOXICITY OF DERRIS ROOT

By J. T. MARTIN, B.Sc., Ph.D.

Department of Insecticides and Fungicides, Rothamsted Experimental Station, Harpenden, Herts

(With 2 Text-figures)

During the past six years, different species and varieties of derris root have been examined chemically and biologically in this Department with a view to obtaining a chemical method for the assessment of their insecticidal activities. The methods used and the results obtained have been reported in a series of communications (Tattersfield & Martin, 1935, 1936, 1937, 1938; Martin & Tattersfield, 1936; Martin, 1940).

When tested upon *Aphis rumicis* in a saponin-alcohol medium, rotenone and a deguelin concentrate were found to be appreciably more toxic than were the hydroxyl derivatives, sumatrol and the toxicarol precursor. A chemical separation of the resins was devised, by which the rotenone, deguelin concentrate and sumatrol and toxicarol fractions could be obtained, and their relative toxicities determined. It was then possible to assess the active principles present in any root or resin in terms of rotenone. The resulting 'rotenone equivalent' percentage of the root or resin, obtained by the summation of the percentage of rotenone with one-fifth of the percentage of the deguelin concentrate and one-fifteenth of the percentage of the alkali-soluble (sumatrol and toxicarol) fraction, was found to give a satisfactory assessment of the biological activities of certain roots or resins to two species of insect, *A. rumicis* L., and *Oryzaephilus surinamensis* L. The satisfactory relationship between rotenone equivalent and toxicity was found to hold for derris roots of widely varying nature, ranging from the Kinta (Sumatra) type root of low rotenone content in proportion to total extractives to the *Derris elliptica* Changi root containing a high proportion of rotenone to other active principles (Martin, 1940).

It has been considered worth while to determine to what extent the relationship between rotenone equivalent and toxicity held for the roots examined and reported upon earlier in this series. In this work, observations upon the stability of rotenone in ground derris root when kept at room temperatures in tins have been recorded. The results are given in this paper.

Until recently, three of the active principles known to occur in derris had been obtained in the crystalline state. These were rotenone, known for many years, sumatrol, isolated by Cahn & Boam (1935), and the toxicarol precursor, first separated by Tattersfield & Martin (1937). The deguelin concentrate fraction of derris may account for as much as 55 % of the total extractives, and with an activity of approximately one-fifth that of rotenone, plays an

important part in the toxic effect of the root. Information has been needed upon the constitution of this fraction, and in this progress has lately been made. Meyer & Koolhaas (1939*a*), from an unspecified type of root, and Harper (1939*a*), from *D. elliptica*, have reported the isolation of a new substance, termed by Harper '*l*-elliptone'. Meyer & Koolhaas (1939*b*) report tests by Van der Vecht indicating that elliptone (or as they term it 'derride') has a toxicity to caterpillars comparable with that of rotenone. Harper (1939*b*) has established the structure of elliptone, which under the fractionation method employed in the determination of the 'rotenone equivalent' remains in the deguelin concentrate. The corresponding phenolic compound, malaccol, has also been isolated (Meyer & Koolhaas, 1939*a*; Harper, 1940). Optically active deguelin has yet to be separated from the deguelin concentrate, but, in the light of present knowledge, it is likely to account for probably not more than half of this fraction. (The term 'deguelin concentrate' will, however, be retained at present on grounds of familiarity.) It is of importance, as an aid to our understanding of the toxic action of the deguelin concentrate fraction of derris root, to know the toxicities of constituent compounds as they become available. The toxicity of *l*-elliptone relative to that of rotenone has now been determined. The insecticidal activity of malaccol remains to be found.

The assessment of the toxicities of different varieties of root by the determination of their rotenone equivalents is based upon the summation of the toxicities of the three groups of active principles, rotenone, deguelin complex and the alkali-soluble constituents, with allowances for the extents to which they are present. This method will only prove valid if the toxic properties of the groups are truly additive. In other words, the rotenone equivalent method of assessing toxicity is likely to break down if there is an appreciable augmentation or depression of the toxic action of one individual or group of active principles by another. Bliss (1939) claims to have detected evidence of such augmentation of toxicity (synergism) among the active principles of derris, using data published by ourselves. Preliminary work on the action of certain active principles of derris when applied jointly has now been carried out, and the results have been examined statistically. Further examination of the data, with a consideration of the theoretical aspects of the detection and expression of synergistic or antagonistic action, has been made by Finney (1942).

The mortality figures given throughout this paper are those after allowing for the mortality in control tests. A probability level of 5 % has been used in determining significance in the statistical analyses. At this level χ^2 for $n=1$ is 3·841, for $n=2$ is 5·991, for $n=3$ is 7·815 and for $n=4$ is 9·488. The calculated regression lines are given in Figs. 1 and 2.

Chemical and biological tests

The correlation between the rotenone equivalents and the toxicities of roots examined previously

During 1935 and 1936, a number of roots, ranging from the Sumatra-type to a high-grade *D. elliptica* root, were examined chemically and biologically. Of these, four roots, *D. malaccensis* Sarawak erect (our ref. No. W. 151), *D. polyantha* (probably *elliptica*) (W. 150), *D. elliptica* from Paya Lebar (W. 153) and a 'Sumatra-type' root (W. 170), were selected for further examination in February 1939, with a view to determining the correlation between their rotenone equivalents and toxicities.

Ethyl acetate extraction of the roots was made, and the resins were fractionated by the method for the determination of the rotenone equivalent given by Martin (1940, p. 289). The results are shown in Table 1.

The roots were tested biologically against *Aphis rumicis*, using an alcohol-saponin medium, during 1934 and 1935. The *Derris malaccensis* root (W. 151) was taken as standard, and was tested on separate

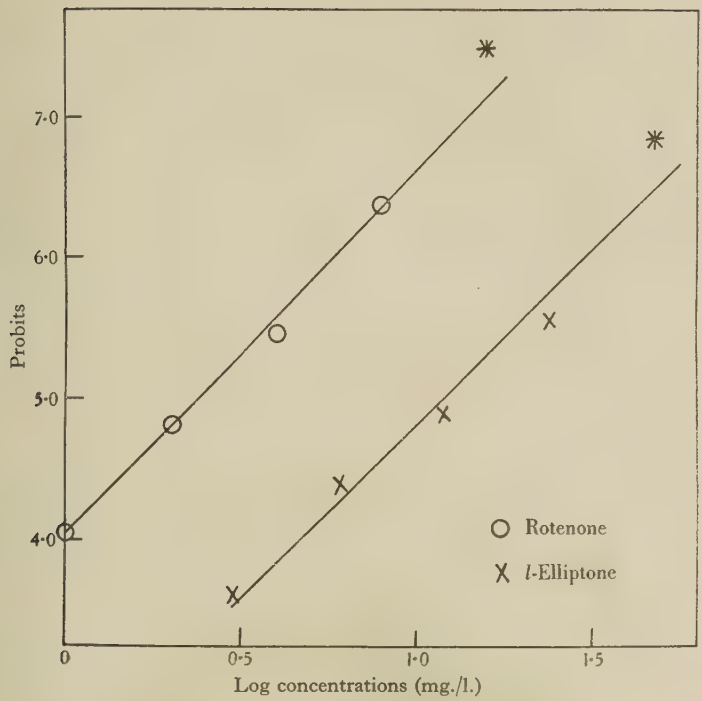

Fig. 1. The toxicity of *l*-elliptone relative to that of rotenone.

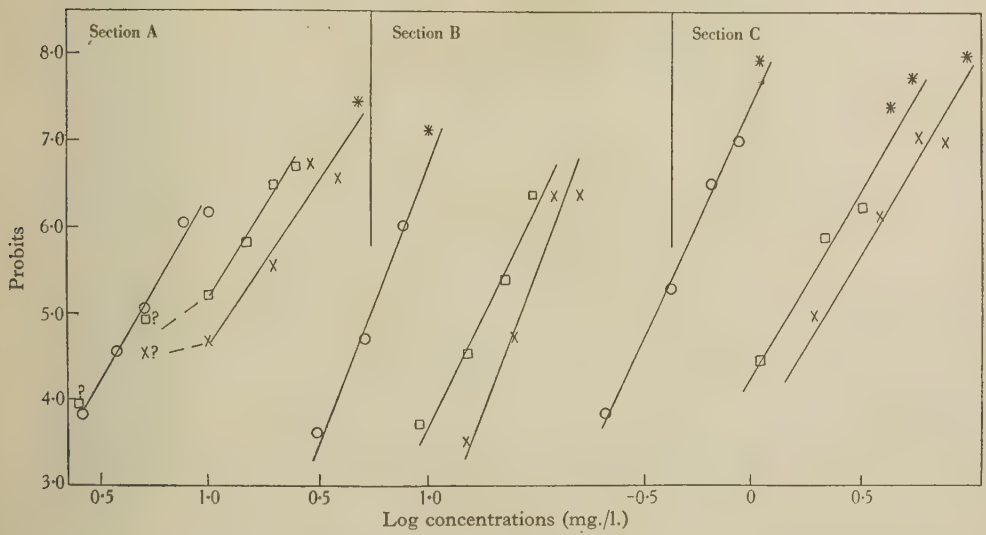

Fig. 2. The toxicities of rotenone, a deguelin concentrate, *l*-elliptone and *l*-α-toxicarol separately and as mixtures.

days against the remaining three roots. The comparison of W. 151 with W. 170 was made on 12 August 1935, with W. 150 on 20 July 1934, and with W. 153 on 30 July 1934. The relevant data for the comparison of W. 151 with W. 170 are given by Martin & Tattersfield (1936, p. 884), and those for the comparisons of W. 151 with W. 150 and W. 153 by Tattersfield & Martin (1935, pp. 593 and 596 respectively). The concentrations of air-dried root in mg./l. given in these communications have been corrected to an oven-dried basis by calculation from the moisture contents of the roots at the times of the biological tests (W. 170, 6·7; W. 151, 6·4; W. 150, 6·5; and W. 153, 5·5 %).

TABLE 1. *Fractionation of derris resins*, W. 170, W. 151, W. 150 *and* W. 153

	% of oven-dry root			
	Sumatra-type W. 170	*D. malaccensis* W. 151	*D. polyantha* W. 150	*D. elliptica* W. 153
Total extract $=A$	21·1	21·3	14·0	28·0
Neutral resin $=B$	6·9	11·9	11·6	23·7
Rotenone from CCl_4 complex	0·7	2·36	4·15	9·21
% purity of complex by rotation	89·0	91·6	94·8	94·5
Purified rotenone $=R$	0·63	2·16	3·93	8·71
Deguelin concentrate $B-R=D$	6·3	9·7	7·7	15·0
Toxicarol fraction $A-B=T$	14·2	9·4	2·4	4·3
Rotenone equivalent $=R+D/5+T/15$	2·84	4·73	5·63	12·00

TABLE 2. *The relative toxicities of* W. 170, W. 151, W. 150 *and* W. 153 *derris roots*

On basis of % $B+M+D$ insects

	W. 170		W. 151
χ_a^2	5·397 ($n=3$)		5·006 ($n=3$)
χ_b^2		0·016	
M		0·350	
Relative potency (antilog M)	1·0		2·24
S.E. of M		±0·022	
S.E. of relative potency		±0·11	
M.L.D. of root (mg./l.)	712		318
	W. 151		W. 153
χ_a^2	0·764 ($n=3$)		4·776 ($n=4$)
χ_b^2		0·142	
M		0·342	
Relative potency (antilog M)	1·0		2·20
S.E. of M		±0·019	
S.E. of relative potency		±0·096	
M.L.D. of root (mg./l.)	280		94

On basis of % $M+D$ insects

	W. 151	W. 150
Relative potency	1·0	1·1
M.L.D. of root (mg./l.)	269	240

On basis of % $S+M+D$ insects

	W. 151	W. 150
Relative potency	1·0	1·2
M.L.D. of root (mg./l.)	204	170

The data for each regression line may be regarded as not heterogeneous while in no comparison do the regression lines depart significantly from parallelism.

In assessing the toxic effect, the percentages of badly affected (B), moribund (M) and dead (D) insects were taken in the comparison of the toxicities of W. 170 and W. 153 with that of W. 151. This is in accordance with our latest procedure in the biological examination of derris roots (see Martin, 1940, p. 276). The assessment of toxicity in the comparison of W. 151 with W. 150, however, was made in two ways, by determining the percentages of moribund and dead insects, and of the slightly

affected (*S*), moribund and dead insects. The results available for the toxicity of W. 150 relative to that of W. 151 are not therefore strictly comparable with those for the comparisons of W. 170 and W. 153 with W. 151. If, however, in the comparisons of W. 150 and W. 151, the assessment of toxicity had been made on the basis of the percentages of badly affected, moribund and dead insects, it is probable that a value for the relative toxicity of W. 150 and W. 151 between the values obtained on the bases of moribund and dead insects, and of slightly affected, moribund and dead insects would have resulted.

The figures obtained in the comparison of the toxicities of W. 170 and W. 153 relative to that of W. 151 were subjected to statistical analysis by the method of Bliss (1935) an example of which has previously been given (Appendix by Cochran to the paper by Tattersfield & Martin, 1938). The relative toxicity of W. 150 and W. 151 on the two bases on which assessment of toxic effect was made, has been determined from the freehand regression lines. The results are shown in Table 2.

The effect of the change from one time to another in insect resistance is seen in the concentrations of the W. 151 root needed to give a 50 % toxic effect in the insects. From Table 2, it is seen that the W. 170 root is slightly less than half (× 0·45), while the W. 153 root is × 2·2 as toxic as the W. 151 root. On both the bases of assessment of toxicity used, the W. 150 root is slightly more toxic (× 1·1 to 1·2) than the W. 151 root.

Taking the *D. malaccensis* (W. 151) root as standard, the relative toxicities, determined biologically (Table 2) and calculated from the ratios of the chemically determined rotenone equivalents (Table 1), are shown in Table 3.

The agreement between the relative toxicities of these extreme types of root determined by the two methods, as with the roots previously reported (Martin, 1940) is again good.

TABLE 3. *The relative toxicities of derris roots determined biologically and chemically*

	Relative toxicities determined	
	Biologically	From rotenone equivalent
Sumatra-type W. 170	0·5	0·6
D. malaccensis W. 151	1·0	1·0
D. polyantha W. 150	1·1–1·2	1·2
D. elliptica W. 153	2·2	2·5

TABLE 4. *Total extractives of derris roots*

Year	Solvent	W. 170	W. 151	W. 150	W. 153
1933	Ether	—	19·9	12·3	25·4
1935	Ether	19·6	19·8	—	—
1935	Benzene	20·4	20·8	—	—
1939	Ethyl acetate	21·1	21·3	14·0	28·0

The stability of the resins in air-dried ground derris roots stored in tins

The biological determinations of toxicity reported above were made some 4–5 years before the determination of the rotenone equivalents. In this case, before the value of the rotenone equivalent as an index of potency can be assessed, it is important to know whether or no the derris resins have changed in character during the intervening years. Certain chemical determinations made in 1933–6, namely, those of total extracts, of rotenone and of rotenone plus deguelin (Gross & Smith, 1934) contents and of the fractions soluble in ether and extractable by potash on alkaline fractionation of an ether solution of the resins, may be used in checking this. The yields of dehydro compounds obtained earlier are of little value, as, particularly with toxicarol, they tend to be low. The values reported in the earlier communications on an air-dried root basis have been corrected to an oven-dried basis to render them comparable with the values given in this paper.

(*a*) *Total extract.* In 1934 and 1935, ether and benzene extractives were determined on the roots under consideration. The values obtained are compared in Table 4 with those for total extractives obtained in 1939 using ethyl acetate as solvent.

It is known that ethyl acetate extracts from derris root slightly larger quantities of resinous material than does ether. There has thus been no diminution in the contents of extractives over a period of 4–5 years.

(*b*) *Rotenone.* During the period under review, the rotenone contents of the roots have been determined by different methods. The values obtained are tabulated in Table 5.

There is no indication of any material change in the rotenone contents of the roots on keeping the air-dried ground powders in tins at room temperatures (16–20° C.) for the period of 6 years.

(*c*) *Rotenone plus deguelin* (*Gross & Smith*). Determinations of the rotenone plus deguelin contents of three of the roots were made in 1933 by the method of Gross & Smith. The results given by Tattersfield & Martin (1935), although of an approximate nature, show a definite relationship to the values obtained in 1939 for the summation of the purified rotenone and deguelin concentration fractions resulting in the determinations of the rotenone equivalents (Table 6).

(*d*) *Percentages of resins extractable by alkali.* During 1935, the resins from 5 g. each of the W. 170 and W. 151 roots, in ether solution, were extracted with 5 % aqueous potash, and the fractions of the resins remaining in the ether and extracted by the potash were determined. The results are given by Martin & Tattersfield (1936, p. 890). These are compared, in Table 7, with the results obtained in the alkaline fractionation method used in 1939 for the determination of the rotenone equivalents.

TABLE 5. *Purified rotenone contents of derris roots*

Year	Method	W. 170	W. 151	W. 150	W. 153
1933	Ether, CCl_4, recrystallization from alcohol	—	1·96	3·99	9·00
1935	Ether, CCl_4, addition of rotenone, recrystallization from alcohol	—	1·93	—	—
1936	Ether, CCl_4, recrystallization from CCl_4	0·50	2·00	—	—
1936	Ether, CCl_4, alcohol trituration	0·58	2·06	—	—
1936	Ether, potash extraction, CCl_4	0·43	1·82	—	—
1939	Ethyl acetate, potash extraction of ether-benzene solution, CCl_4	0·63	2·16	3·93	8·71

TABLE 6. *Rotenone and deguelin contents by the Gross & Smith* (1934) *method compared with the rotenone and deguelin concentrate fractions obtained in the determination of the rotenone equivalents*

Year		W. 151	W. 150	W. 153
1933	Rotenone plus deguelin (Gross & Smith)	10	7·5–10	15–20
1939	Purified rotenone + deguelin concentrate	11·9	11·6	23·7

TABLE 7. *Fractionation of the resins of* W. 170 *and* W. 151 *by means of potash*

		% of oven-dried root	
Year		W. 170	W. 151
1935	Ether extract	19·6	19·8
1939	Ethyl acetate extract	21·1	21·3
1935	Resin soluble in ether after potash extraction	4·2	9·6
1939	Resin soluble in benzene-ether after potash extraction	6·9	11·9
1935	Resin extracted by potash	14·4	9·3
1939	Resin extracted by potash	14·2	9·4

There has been no change in the fractions of the resins extractable by potash over a period of 4 years. A less exact relationship holds for the rotenone plus deguelin concentrate fractions of the total extracts remaining in solution in ether or in ether-benzene after potash extraction. The lower values obtained in 1935 for the fractions of the resins remaining in ether after potash extraction may well be a reflexion of the smaller amounts of resin extracted by ether than by ethyl acetate.

It is clear, from a consideration of sections (*a*), (*b*), (*c*) and (*d*), that the natures of the resins of the W. 170 and W. 151 roots have changed but little, if at all, in respect of total extractives, rotenone content and of the fractions separated by alkali during the period 1935 to 1939. Similarly, the W. 150 and W. 153 roots have shown no significant falling off in total extractives or rotenone contents during this period.

The fact that the ground air-dried roots have shown little change in rotenone content, detectable by chemical means, over a period of 6 years (see Table 5) is of particular interest.

Biological tests

The toxicity of l-*elliptone relative to that of rotenone.* The high melting form of *l*-elliptone (m.p. 168° C., $[\alpha]_D^{20}$ in benzene $-18°$), separated by Dr S.H. Harper from the neutral resin of *Derris elliptica*, was used in these tests. The chrysanthemum aphis, *Macrosiphoniella sanborni*, reared upon chrysanthemum plants in a cool glasshouse, was used as test insect. The results were as given in Table 8.

Table 8. *Toxicity of* l-*elliptone. Tests of 2 Jan. 1940. Fivefold replication. Results one day after spraying. Medium 0·5 % saponin, containing 10 % of alcohol. Tattersfield apparatus*

	Conc. mg./l.	No. of insects used	% mortality ($B+M+D$)	% S.E. ±
Rotenone	16	51	100	—
	8	48	91·5	4·3
	4	47	67·6	14·2
	2	48	42·7	13·6
	1	48	17·2	9·2
l-Elliptone	48	49	100	—
	24	49	70·9	8·7
	12	49	45·9	10·2
	6	49	27·2	15·5
	3	49	8·4	3·5
Controls (alcohol-saponin)	—	50	2·0	—

The data with the calculated regression lines are plotted in Fig. 1.

On analysis the following values were obtained:

χ_a^2 Rotenone 1·097 ($n=3$)

l-Elliptone 4·117 ($n=3$)

The data for each line are not heterogeneous.

χ_b^2 0·219

The regression lines do not depart significantly from parallelism.

$M=0·694 \pm 0·054$

Relative potency (antilog M) $=4·94 \pm 0·61$.

Rotenone is thus $\times 5$ as toxic as *l*-elliptone to *Macrosiphoniella sanborni* when tested in an alcohol-saponin medium.

The toxicity of poisons applied jointly

Experiments have been carried out to determine to what extent the observed toxicity of a mixture compares with the predicted value calculated from the individual toxicities of the constituent poisons. Information on this point is required in order to establish the validity of the rotenone equivalent method of assessing the toxicities of derris roots and resins. An appreciable, if unpredictable augmentation or depression of the toxic effect of one constituent by the action of another might well invalidate the assessment of toxicity by chemical means.

The correlation between the observed and predicted toxicities of a mixture of rotenone and of a deguelin concentrate. The preparation of the deguelin concentrate from a *Derris elliptica* Changi root is described by Martin (1940, p. 285). Biological trials were carried out with

pure rotenone and with this preparation, using *Macrosiphoniella sanborni*. Probit-log concentration lines were determined for rotenone and the deguelin concentrate separately and for a mixture containing one part of rotenone to four parts of the deguelin concentrate. The proportions were chosen as it had previously been shown (Tattersfield & Martin, 1938) that the rotenone-free resin from *Derris elliptica* was likely to be of the order of one-fifth as toxic as pure rotenone.

The toxicity results are given in Table 9.

TABLE 9. *The toxicities of rotenone, a deguelin concentrate, and of a mixture. Tests of* 17 *Nov.* 1938. *Fivefold replication. Results one day after spraying. Medium* 0·5 % *saponin, containing* 5 % *of alcohol. Tattersfield apparatus*

Concentrations (mg./l.)		No. of insects used	% mortality $(B+M+D)$	% S.E. ±
Rotenone	Deguelin concentrate			
10·2	—	50	88·0	5·4
7·7	—	49	85·7	6·2
5·1	—	46	52·2	8·1
3·8	—	48	33·3	6·4
2·6	—	50	12·0	8·1
—	50·5	48	100	—
—	40·4	50	94·0	6·0
—	30·3	49	95·9	4·6
—	20·2	48	70·8	8·9
—	10·1	48	37·5	8·9
—	5·1	49	32·6	6·2
5·1	20·3	50	96·0	2·4
4·0	16·3	46	93·5	2·9
3·0	12·2	48	79·2	10·2
2·0	8·1	46	58·7	5·0
1·0	4·1	46	47·8	11·2
0·5	2·0	47	14·9	6·6
Controls (alcohol-saponin)	—	49	0	—

The data are plotted in Fig. 2A.

The rotenone toxicity values, even to the lowest concentrations tested, are closely fitted by a straight line. In the lines for the deguelin concentrate and for the mixture, however, there are breaks, the lowest concentration of the deguelin concentrate and the two lowest concentrations of the mixture tested appearing more toxic than the rest of the data would indicate. The lowest determinations for both the deguelin concentrate and the mixture, with standard errors of ±6·2 and ±6·6 % respectively, appeared, however, to be perfectly valid. The second lowest point for the mixture was more open to suspicion. The comparatively high mortality (47·8 %) obtained in this determination was largely due to an unexplained high kill in one of the five replicates, resulting in an appreciable standard error of ±11·2 %. Omitting this replicate, the percentage mortality for the point, determined using 36 insects, was reduced to 36·1 with a standard error of ±3·7 %. The value then fell more closely in line with the remainder of the data for the mixture, but a slight break in the regression line was still apparent.

It is significant that no break occurs in the regression line for the *pure* substance rotenone, but occurs in the case of the deguelin concentrate (itself a mixture) and of the mixture of this with rotenone. Breaks of this nature in dosage-mortality lines have been discussed by

Bliss (1939) who considers it likely that the lower, flatter portion of the line is determined by the less active component of a mixture.

Inclusion of all the points obtained rendered the data for the deguelin concentrate heterogeneous (χ_a^2 11·269 for $n=4$), and caused the regression lines for the deguelin concentrate and mixture to depart significantly from parallelism with that of the rotenone (χ_b^2 for rotenone and deguelin concentrate lines 5·163), although the deguelin concentrate and mixture lines themselves did not depart significantly from parallelism (χ_b^2 0·677).

It was therefore decided to confine the investigation of synergistic or antagonistic action to the upper portions of the lines. The lowest point of the deguelin concentrate and the two lower points of the mixture (marked in Fig. 2 A ?) were therefore omitted from the calculations.

The data for each line were then found to be not heterogeneous, with no significant departure of the regression lines from parallelism:

χ_a^2	Rotenone	1·742 ($n=3$)
	Deguelin concentrate	4·470 ($n=3$)
	Mixture	0·379 ($n=2$)
χ_b^2	Rotenone and deguelin concentrate	0·026
	Rotenone and mixture	0·012

Using parallel probit lines fitted to all three sets of data, the observed doses (mg./l.) required for 84 % mortality of the insects (6 probits) were as follows:

Rotenone	8·67
Deguelin concentrate	23·6
Mixture	16·1

Reducing the mixture to its rotenone equivalent, the predicted dose, calculated on the hypothesis of similar action, is 17·5 mg./l. The mixture proved to be 8·6 % more toxic than would be predicted from the potencies of its constituents alone. This slight apparent synergistic effect, however, is not significant.

Full details of the methods and computations involved in the determination of the predicted dose of the mixture and of the significance of effect are given by Finney (1942).

The correlation between the observed and predicted toxicities of a mixture of rotenone and l-*elliptone.* In view of the break in the regression line for the deguelin concentrate it was decided next to use, in combination with rotenone, one of the components of this fraction (elliptone) which had been obtained crystalline. The high melting form, m.p. 168° C. and $[\alpha]_D^{20}$ in benzene $-18°$, was used. The test insect was again the chrysanthemum aphis, *Macrosiphoniella sanborni*. The toxicity data are given in Table 10.

It will be noted that with the crystalline compounds rotenone and *l*-elliptone, there is no break in either line, the lowest values being in accordance with the rest of the data (Fig. 2 B).

On analysis, the following results were obtained:

χ_a^2	Rotenone	1·833 ($n=2$)
	l-Elliptone	4·447 ($n=2$)
	Mixture	2·521 ($n=2$)
χ_b^2	Rotenone and *l*-elliptone	0·002
	Rotenone and mixture	1·595

The data for each line are not heterogeneous, neither do the lines depart significantly from parallelism.

The relative toxicity of rotenone and *l*-elliptone was determined statistically as follows:

$$M = 0{\cdot}696 \pm 0{\cdot}028.$$

Rotenone is $\times 4{\cdot}97 \pm 0{\cdot}32$ more toxic than *l*-elliptone. This value is in close agreement with that reported earlier (p. 75).

The observed median lethal doses (mg./l.) were as follows:

Rotenone	5·34
l-Elliptone	26·51
Mixture	17·80

TABLE 10. *The toxicities of rotenone, l-elliptone and of a mixture. Tests of 7 July 1941. Five-fold replication. Results one day after spraying. Medium 0·5 % saponin, containing 5 % of alcohol. Tattersfield apparatus*

Conc. (mg./l.)		No. of insects used	% mortality ($B+M+D$)	% S.E. ±
Rotenone	*l*-Elliptone			
10·2	—	28	100	—
7·65	—	40	84·7	8·6
5·10	—	48	38·4	14·1
3·06	—	49	8·4	4·5
—	50·5	51	91·9	5·8
—	37·9	49	91·6	3·7
—	23·3	49	39·6	6·8
—	15·2	46	6·9	4·2
5·1	25·3	50	91·8	2·0
3·8	18·9	48	66·0	4·0
2·55	12·6	44	32·8	9·9
1·5	7·6	49	10·4	4·0
Controls (alcohol-saponin)	—	48	2·1	—

The data are plotted in Fig. 2B.

Fitting parallel lines to the sets of data as before, and working on the basis of similar action, the predicted median lethal dose for the mixture was 15·96 mg./l. The mixture was 10·3 % less toxic than predicted from the potencies of its constituents. This apparent antagonistic effect was, however, not significant.

The correlation between the observed and predicted toxicities of a mixture of rotenone and l-α-toxicarol. So far, no evidence of a significant augmentation or depression of toxic action has been observed in tests using rotenone in admixture with a deguelin concentrate or with *l*-elliptone, a crystalline constituent of the deguelin concentrate fraction of derris resin. There remains, however, the possibility that toxicarol (hydroxy-deguelin) may accentuate the toxicity of rotenone. We already have evidence, to be published later, that certain hydroxyl compounds will, even in small quantities, appreciably augment the insecticidal potency of rotenone. The determination of the effect of *l*-α-toxicarol therefore became of importance.

Tests were made, again using *Macrosiphoniella sanborni* as test insect, with rotenone in admixture with pure *l*-α-toxicarol. This was prepared by Dr S. H. Harper, and was free from the substance of m.p. 219° and methoxyl content of 22·6 % contaminating earlier

preparations of *l*-α-toxicarol. It showed $[\alpha]_D^{20}$ in benzene $-66{\cdot}8°$ and a methoxyl content of 15·1 %. The preparation of this pure *l*-α-toxicarol has been described by Harper (1940).

Toxicity tests of rotenone and *l*-α-toxicarol separately and in admixture of 1 part of rotenone with 9·2 parts of *l*-α-toxicarol gave the following results (Table 11).

Probits are plotted against the logarithms of the concentrations used in Fig. 2 C. The points for rotenone, *l*-α-toxicarol and the mixture are closely fitted by straight lines even to the lowest concentrations used.

TABLE 11. *The toxicities of rotenone,* l-α-*toxicarol and of a mixture. Tests of* 24 *September* 1941. *Fivefold replication. Results one day after spraying. Medium* 0·5 % *saponin, containing* 5 % *of alcohol. Tattersfield apparatus*

Conc. (mg./l.)				
Rotenone	*l*-α-Toxicarol	No. of insects used	% mortality ($B+M+D$)	% S.E. ±
1·06	—	51	100	—
0·85	—	48	97·9	2·0
0·64	—	48	93·8	2·8
0·42	—	48	62·5	11·2
0·21	—	48	12·5	4·6
—	9·75	49	100	—
—	7·80	48	97·9	2·0
—	5·85	52	98·1	2·0
—	3·90	49	87·7	4·9
—	1·95	48	50·0	7·4
0·53	4·88	48	100	—
0·42	3·90	48	100	—
0·32	2·93	49	89·8	5·5
0·21	1·95	50	82·0	9·1
0·11	0·98	50	30·0	7·1
Controls (alcohol-saponin)	—	50	0	—

The data for each line may be regarded as not heterogeneous, χ_a^2 for $n=3$ in each case being

Rotenone	0·665
l-α-Toxicarol	3·120
Mixture	3·304

neither do the regression lines depart significantly from parallelism:

$$\chi_b^2 \qquad 3{\cdot}678\ (n=2).$$

The relative potency of rotenone and the pure *l*-α-toxicarol was determined as follows:

$$M = 0{\cdot}789 \pm 0{\cdot}038.$$

Rotenone is thus ×6·15 more toxic than the pure *l*-α-toxicarol.

The observed median lethal doses (mg./l.) were as follows:

Rotenone	0·34
l-α-Toxicarol	2·09
Mixture	1·44

The predicted median lethal dose for the mixture, using as before a common slope of the regression lines and working on the basis of similar action, was 1·39 mg./l. The mixture proved to be 3·5 % less toxic than predicted from the potencies of rotenone and *l*-α-toxicarol, but this slight antagonism was not significant.

Discussion

In our earlier attempts to assess the insecticidal potencies of derris roots by chemical means a number of determinations, rotenone, ether extract, methoxyl content, dehydro compounds and ether-soluble resin after potash treatment were used, but were found to be inadequate either separately or in combination. Trouble was experienced in assessing the toxicities of roots of different species due to the varying proportions in the roots of the three main classes of the active principles, rotenone, the non-hydroxyl compounds other than rotenone (of which *l*-elliptone may be cited as an important example) and the hydroxyl compounds (of which *l*-α-toxicarol appears to be the most important), each with different biological activities. The introduction of the rotenone equivalent method of assessing potency (Martin, 1940) by which the full toxic effect of the root was determined by the summation of the toxicities of each fraction with allowances for the extents to which they occurred, resulted in a satisfactory evaluation of roots of widely differing type. The method has now been applied successfully to four of the roots examined earlier in our work, for which other chemical determinations had been found to be unsatisfactory as an expression of potency.

It was realized that any chemical method of assessing toxic action might well be invalidated if there existed an appreciable, unpredictable synergistic or antagonistic action between the active principles, or between the active principles and the inactive resin. This possibility has now been tested, using, in comparison with rotenone, *l*-elliptone as representative of the group of residual non-hydroxyl compounds and pure *l*-α-toxicarol as representative of the class of toxic hydroxyl constituents of derris root. No significant synergistic or antagonistic action using the pure active principles has been found. The possibility that the presence of the inert fraction of the resin (i.e. fatty material devoid of active principles) may result in the augmentation or depression of the effect of the active principles has already been examined (Martin, 1940). No change in the potency of rotenone was found on admixture with an inert fraction of the resin obtained from *Derris malaccensis*. The toxicities of the active principles may therefore be considered to be additive in character, and uninfluenced by the presence of inactive resin. This fact provides justification for the rotenone equivalent method of assessing the toxicities of derris roots or resins.

The determined potency of *l*-elliptone (one-fifth that of rotenone) places this compound second in importance to rotenone among the naturally occurring active principles so far separated from derris root. The isolation and determination of the structure of *l*-elliptone are important steps forward in an understanding of the nature of the deguelin concentrate fraction of derris resin. In this connexion, the author isolated, in May 1940, yellow crystals from the deguelin concentrate of a *D. malaccensis* root. These melted in the region of 187–193° C., gave a positive Durham test, contained 15·65 % of methoxyl, and on elementary analysis proved to be isomeric with rotenone. Yellow crystals of melting point 164° C. were also obtained. Both compounds showed an appreciable toxicity to *Macrosiphoniella sanborni*, but further work on their nature is required.

An appreciable difference was found in the median lethal doses of rotenone alone in the experiments of 7 July and 24 Sept. 1941. Although a change in insect resistance may have played a part, the discrepancy was attributed to the use in the second experiment of a fresh and more efficient preparation of saponin.

In the third experiment on the effect of poisons applied jointly the toxicity to *M. sanborni* of pure *l*-α-toxicarol (showing the theoretical methoxyl content of 15·1 %) was found to be approximately one-sixth that of rotenone. This preparation is thus appreciably more toxic than the earlier preparations of the toxicarol precursor tested against *Aphis rumicis* (Tattersfield & Martin, 1938). This brings the toxicity of pure *l*-α-toxicarol, relative to that of rotenone, more into line with the toxicities of *l*-elliptone and of the deguelin concentrate.

Summary

The assessment of toxicity by the determination of the rotenone equivalent has been shown successfully to apply to four of the derris roots examined earlier in this series of investigations. Observations on the stability of the resins in ground roots stored in tins at room temperatures have been recorded. *l*-Elliptone has been shown to be one-fifth as toxic as rotenone to *Macrosiphoniella sanborni* when tested in an alcohol-saponin medium. The toxicities of poisons applied jointly have been examined. The observed toxicities of mixtures of rotenone with a deguelin concentrate, *l*-elliptone and *l*-α-toxicarol have been compared with those predicted from the potencies of the constituent poisons. No significant synergistic or antagonistic effect has been found. The bearing of this upon the validity of the rotenone equivalent method of assessing toxicity has been discussed.

I wish to express my indebtedness to Mr D. J. Finney for his helpful collaboration, and to Dr C. Potter for assistance in the biological tests.

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THE ANALYSIS OF TOXICITY TESTS ON MIXTURES OF POISONS

By D. J. FINNEY

Rothamsted Experimental Station, Harpenden, Herts

(With 1 Text-figure)

The development of probit analysis for the statistical treatment of data from toxicity tests, when these data are obtained in the form of percentage kills at a series of dosages, has its roots in papers by Gaddum (1933) and Bliss (1935 *a*, *b*). Bliss (1935*b*) discussed the estimation of the relative potencies of poisons from the results of parallel tests on a series of dosages. The next step to complete understanding of the mode of action of such poisons should be an investigation of the potencies of their mixtures in varying proportions and of the theoretical basis of such joint action.

Bliss (1939) considered the problem of predicting the toxicity of a mixture of poisons from knowledge of the effects of the constituents separately. He distinguished three types of action, giving examples illustrative of each. He does not appear, however, to have examined very closely the mathematical expressions representing these types of joint action, since these expressions do not stand in any clear logical relationship to one another. The purpose of the present paper is to compare and contrast the three situations, to suggest improvements in their mathematical representation, and to give tests of the adequacy of these as explanations of experimental data. These tests will be illustrated on recent experiments by Martin (1942).

Mathematical models for joint action

The three types of joint action which Bliss has discussed he calls *independent*, *similar* and *synergistic*. Synergistic action, he states, is 'characterized by a toxicity greater than that predicted from experiments with the individual constituents'. Though not treated in any detail by Bliss, the possibility of *antagonistic* action, or negative synergism, in which the toxicity of a mixture is less than is expected, must also be recognized. Cases of antagonism have been described by Clark (1937, ch. 17), and various formulae suggested as fitting them; his examples are, however, of so different a nature that it is scarcely surprising that they do not fall within the scope of Bliss's work or of the present paper. These verbal definitions of synergism and antagonism would appear to include all situations not covered by the other two systems. Yet the mathematical model set up for the quantitative study of synergism is not clearly contrasted with those for independence and similarity, nor can either of the latter be treated as special cases having 'zero synergism'.

In order to test the agreement of various sets of data with these types of joint action, it is necessary to have, in mathematical terms, a clear statement of what they imply.

Independent action

Suppose the amounts of the two constituents of a mixed poison to be such as would give kills of proportions p_1, p_2 respectively of the test organism if they were applied separately.

The constituents are said to act independently if the mortality caused by the mixture is a proportion

$$p = p_1 + p_2 - p_1 p_2. \tag{1}$$

This may easily be extended to cases of three or more constituents. It should be noted that even if the separate components yield linear regressions of probits on dosage or log-dosage, the probits of the kills at different dosages of a mixture of constant proportions will not necessarily lie on a straight line. If the original probit lines are parallel, however, examples given by Bliss indicate that the probits for the mixture will lie very close to a third parallel line.

Similar action

If two poisons show parallel regressions of probits on log-dosages when they are applied separately to the test organism, the probits, Y_1, Y_2, of the percentage kills resulting from dosages z_1, z_2 of the poisons may be predicted from

$$Y_1 = a_1 + b \log z_1, \tag{2}$$

$$Y_2 = a_2 + b \log z_2, \tag{3}$$

b being the common slope of the regression lines. Using the symbol ρ to represent the potency of the second poison relative to that of the first, $\log \rho$, or M in the notation generally adopted, is given by

$$\log \rho = (a_2 - a_1)/b. \tag{4}$$

Any given dose of the second may be converted into its *equivalent dose* (Martin, 1940) of the first poison by multiplying it by ρ; in fact

$$Y_2 = a_1 + b \log (\rho z_2). \tag{5}$$

Suppose now that a mixture contains proportions π_1 and π_2 of the two poisons. A dose z_m of this mixture has as its equivalent dose of the first poison an amount $(\pi_1 + \rho\pi_2)\, z_m$. The two poisons are said to act similarly if the toxicity of the mixture is the same as that of its equivalent dose, that is to say, if the probits obtained for the mixture are satisfactorily fitted by the equation

$$Y_m = a_1 + b \log \{(\pi_1 + \rho\pi_2)\, z_m\}. \tag{6}$$

It may be observed that, if $(\text{L.D. } 50)_1$ calculated from

$$\log (\text{L.D. } 50)_1 = (5 - a_1)/b \tag{7}$$

represents the median lethal dose of the first component, the median lethal dose for any mixture is predicted as

$$(\text{L.D. } 50)_m = (\text{L.D. } 50)_1/(\pi_1 + \rho\pi_2), \tag{8}$$

which is the dose of mixture to which $(\text{L.D. } 50)_1$ is equivalent. For the second poison applied alone $\pi_1 = 0$, $\pi_2 = 1$. The equations (6) and (8) are, of course, the same as (5) and (7) in Bliss's paper.

Clearly the case of three or more components will fit easily into this scheme also. Bliss states that 'Toxic mixtures of this class have a greater expected potency than those in the preceding (independent action) class', and though no proof of this has been given, it appears to be generally the case.

Synergistic action

Bliss proposes two different formulae as representations of synergistic action which, though not equivalent, 'may be indistinguishable when one component is always a small fraction of the other'. The first of these may be written

$$(z_1+z_2)\, z_2 = L, \tag{9}$$

where z_1, z_2 are the quantities of each component present in any mixture giving a particular mortality (the second being the more active ingredient), the constants L and i presumably being dependent on the level of mortality chosen. The application of this formula must, as Bliss states, be restricted to dosages whose concentrations of the second poison exceed a certain minimum, since the formula indicates that mixtures containing a very small amount of this component would be less toxic than the first component alone.

The second formula, which Bliss appears to prefer, is

$$(1+kz_2)\, z_1^j = k', \tag{10}$$

z_1, z_2 being as before, and k, k' and j constants dependent on the selected mortality level, though j apparently is often very close to unity. Again the formula must be restricted with regard to dosage on account of its behaviour when z_1 becomes small. The product kk' is used by Bliss as a measure of synergism, and, in the one example given, its value is found to be substantially the same at different levels of mortality. Unfortunately, however, poisons whose intensity of synergism is zero do not necessarily act *similarly* or *independently*, since neither $k=0$ nor $k'=0$ makes (10) reduce to either of the simpler types of joint action. There seems no reason to suppose that any large group of cases, which are synergistic in the sense that the mixture of poisons is more potent than would be predicted from knowledge only of the separate components, can be fitted by either of the above formulae.

Their lack of symmetry in respect of the dosages of the two components and the restriction in their ranges of applicability are further objections to these expressions. Without examination of data from a number of experimental tests it is scarcely possible to propose models free from these theoretical objections which will nevertheless fit the facts. As a tentative suggestion, a generalization of the formula for similar action might be tried, in which the dose of one component equivalent to a mixture of known constitution contains terms of higher order in π_1 and π_2. Thus one might look for cases of synergistic action in which the probit of the kill from a mixture was given by

$$Y = a_1 + b \log \{(\pi_1 + \rho\pi_2 + \kappa\sqrt{[\rho\pi_1\pi_2]})\, z\}, \tag{11}$$

the notation being as before, with the addition of a 'coefficient of synergism', κ, a zero value of which implies similar action of the two poisons. Both positive and negative values of this coefficient could be interpreted, the latter implying antagonism. A further advantage of such a formula would be that it, like (1) and (6), could be adapted for use with three or more constituent poisons. No data of suitable type for testing the adequacy of (11) have yet been found.

In the sections which follow the modes of joint action described here will be examined in relation to data given by Martin (1942), and to results from earlier sources.

Toxicity of a mixture of rotenone and deguelin concentrate

A test was made on 17 Nov. 1938 of the toxicities of a range of concentrations of rotenone, of deguelin concentrate, and of a mixture containing one part of rotenone to four of deguelin concentrate, the test insect being *Macrosiphoniella sanborni* (chrysanthemum aphis). Omitting the lowest concentration of the deguelin concentrate and the two lower concentrations of the mixture, the inclusion of which made the data heterogeneous (Martin, 1942), the probits of the kills produced by the three poisons were satisfactorily fitted by linear regressions on the log-dosages, and the three lines did not depart significantly from parallelism. In order to test different types of joint action, parallel regression lines were fitted to the first two series of results. These were

$$Y_r = 2{\cdot}305 + 3{\cdot}939 \log x_r, \tag{12}$$

$$Y_d = 0{\cdot}595 + 3{\cdot}939 \log x_d, \tag{13}$$

from which (4) shows that $\rho = 0{\cdot}368$ is the potency of deguelin concentrate relative to that of rotenone. The probit line for the mixture, calculated from the four concentrations tested, is

$$Y_m = 1{\cdot}222 + 3{\cdot}956 \log x_m. \tag{14}$$

Table 1. *Summary of results for mixture of rotenone and deguelin concentrate*

Conc. of mixture mg./l.	Equiv. rotenone mg./l.	No. of insects	No. dead	%* kill	Observed probit	Expected probits: Independent action	Expected probits: Similar action	w for similar action
25·4	12·56	50	48	96·0	6·751	6·248	6·635	11·355
20·3	10·04	46	43	93·5	6·514	5·772	6·245	16·322
15·2	7·52	48	38	79·2	5·813	5·166	5·756	24·737
10·1	4·99	46	27	58·7	5·220	4·347	5·055	29·226

* There were no deaths among the controls.

In Table 1 the observed mortalities for the mixture are compared with predictions made on the two hypotheses of independent and similar action. For the first, the dosages of mixture are divided into their two components (1/5 rotenone, 4/5 deguelin concentrate), and the kill to be expected from each calculated by means of (12) and (13). Thus 25·4 mg. of mixture will contain 5·08 mg. rotenone and 20·32 mg. degulein concentrate; the probits of the kills expected for these separately are 5·087 and 5·748. The percentage kills, 53·5 and 77·3 %, when combined according to (1) indicate a kill of 89·4 % for the mixture, or a probit of 6·248. The four probits expected on the hypothesis of independent action, shown in the seventh column of Table 1, are all substantially less than the corresponding observed values in the preceding column.

Multiplication of the dosages of the mixture by $(\pi_r + \rho\pi_d)$ gives the equivalent dosages of rotenone shown in the second column of Table 1. Equation (12) for these dosages gives the expected probits on the hypothesis of similar action; these are shown in the eighth column. In terms of the dosage of mixture, these satisfy

$$Y'_m = 1{\cdot}101 + 3{\cdot}939 \log x_m, \tag{15}$$

an equation not very different from (14), showing the hypothesis to be in reasonable agreement with the data. A test of the agreement between hypothesis and observation, which probably over-estimates slightly the significance of the discrepancy, can be made by finding

the weights to be associated with the expected probits, multiplying these by the squares of the corresponding discrepancies, and adding to give a χ^2 with four degrees of freedom. The weights, w, are found by entering Table XI of Fisher & Yates (1938) with the expected probits, and multiplying the weighting coefficients by the number of insects tested. It is found that

$$\chi^2_{[4]} = 2{\cdot}21, \tag{16}$$

a value which could easily occur as a result of chance deviations. A similar test for the independent action hypothesis would clearly indicate the significance of the difference, the value of χ^2 being large. In so far as the observed mortalities are slightly higher than those

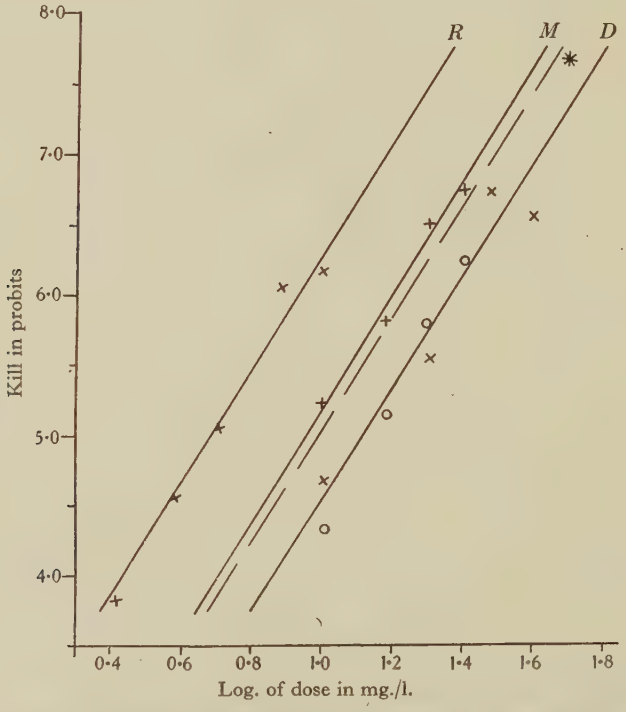

Fig. 1. Toxicity of rotenone and deguelin concentrate to *M. sanborni*. ×, rotenone and deguelin concentrate; +, 1 to 4 mixture; ○, independent action predictions for mixture. Full lines: rotenone (*R*), deguelin concentrate (*D*), mixture (*M*). Broken line: similar action prediction for mixture.

predicted on the basis of similar action, there is a little evidence for synergism, but the data of this experiment can be said to agree well with the hypothesis that rotenone and deguelin concentrate act similarly. The predictions given by the two hypotheses are shown graphically in Fig. 1.

An alternative test may be based on a comparison of predicted and observed median lethal doses for the mixture, though this is applicable only to the hypothesis of similar action. For this purpose it is best to use a regression coefficient, b_c, calculated from all three sets of data, so as to estimate it as accurately as possible. In terms of this, equation (6) may be written

$$Y'_m = \{a_r + b_c \log(\pi_r + \rho\pi_d)\} + b_c \log z_m, \tag{17}$$

while the probit line calculated directly for the mixture is

$$Y_m = a_m + b_c \log z_m. \tag{18}$$

The difference in log L.D. 50 between prediction and observation is

$$M_m = (a_r - a_m)/b_c + \log(\pi_r + \rho\pi_d). \tag{19}$$

It may be shown that the variance of this quantity is

$$V(M_m) = \left[\frac{\pi_r^2}{Sw_r} + \frac{\rho^2\pi_d^2}{Sw_d} + \frac{(\pi_r+\rho\pi_d)^2}{Sw_m} + \{\pi_r\bar{y}_r + \rho\pi_d\bar{y}_d - (\pi_r+\rho\pi_d)\bar{y}_m\}^2 \frac{V(b_c)}{b^2}\right] \Big/ b_c^2(\pi_r+\rho\pi_d)^2, \tag{20}$$

where Sw_r, Sw_d, Sw_m are the totals of the weights and $\bar{y}_r$, $\bar{y}_d$, $\bar{y}_m$ are the mean probits for the three sets of observations. The analogy of (20) with the usual formula for the variance of an M representing the difference in log L.D. 50 between two poisons should be noted. For testing the significance of the departure of M_m from zero, it will generally be sufficient to use a t-test with a standard error derived from (20), but a more exact approach would follow the lines indicated by Cochran (1938).

In the case of the rotenone-deguelin concentrate test, the fitting of parallel probit lines to all three sets of observations gives

$$Y_r = 2{\cdot}304 + 3{\cdot}942 \log z_r, \tag{21}$$

$$Y_d = 0{\cdot}592 + 3{\cdot}942 \log z_d, \tag{22}$$

$$Y_m = 1{\cdot}238 + 3{\cdot}942 \log z_m. \tag{23}$$

From (21) and (22) the potency of deguelin concentrate relative to that of rotenone is given by

$$M = -0{\cdot}434 \pm 0{\cdot}039, \tag{24}$$

or

$$\rho = 0{\cdot}368 \pm 0{\cdot}033, \tag{25}$$

the details of the computations for obtaining the standard errors not being shown here (Bliss, 1935*b*; Cochran, 1938). The median lethal dose for rotenone is 4·83 mg./l., for deguelin concentrate 13·13 mg./l., and for the mixture 8·99 mg./l. Using

$$\pi_r + \rho\pi_d = 0{\cdot}4944, \tag{26}$$

the predicted line for the mixture is obtained as

$$Y'_m = 1{\cdot}098 + 3{\cdot}942 \log z_m, \tag{27}$$

from which, or by equation (8), the predicted median lethal dose is 9·77 mg./l. As a test of the agreement between this value and that determined directly from (23)

$$M_m = -0{\cdot}0357 \pm 0{\cdot}0361. \tag{28}$$

The mixture thus proved 8·6 % more toxic than would be predicted from the behaviour of its constituents alone, but this slight synergistic effect is not significant.

Toxicity of a mixture of rotenone and elliptone

A mixture of rotenone and elliptone, in the proportions of one to five, was subjected to an exactly similar test on 7 July 1941. The probit lines for the two substances separately and that for the mixture did not depart significantly from parallelism, though the slope of the latter was quite substantially less than those of the first two. (The separate regression coefficients were 6·45 and 6·17 for the pure substances, 4·85 for the mixture.) Fitting parallel lines to the data for rotenone and elliptone separately it was found that $\rho = 0{\cdot}201$, and, in

the same manner as in the preceding section, predicted probits for the mixture on the two hypotheses of independent and similar action were obtained. It is obvious from the results summarized in Table 2 that the actual observations for the data do not agree with the independent action predictions, but that the discrepancies are very much less for similar action.

TABLE 2. *Summary of results for mixture of rotenone and elliptone*

Conc. of mixture mg./l.	Equiv. rotenone mg./l.	No. of insects	%* kill	Observed probit	Expected probits: Independent action	Expected probits: Similar action	w for similar action
30·4	10·14	50	91·8	6·392	5·496	6·750	9·692
22·7	7·57	48	66·0	5·412	4·516	5·950	21·842
15·2	5·07	44	32·8	4·555	3·261	4·854	27·770
9·1	3·04	49	10·4	3·741	1·761	3·455	12·489

* Allowing for mortality in controls.

Computing χ^2 as before, $\chi^2_{[4]} = 11{\cdot}06$ (29)

for the agreement of observation with similar action, a value which would be exceeded as a result of chance variations in only about 3 % of cases. This χ^2 appears likely to overestimate the significance of the discrepancy; there is some evidence for antagonistic action of the poisons, as in three cases out of four the observed probits are less than the expected, but the large value of χ^2 is mainly due to the lesser slope of the regression for the mixture.

If parallel regression lines are fitted to the three sets of data, they are found to be

$$Y_r = 0{\cdot}796 + 5{\cdot}779 \log z_r, \quad (30)$$

$$Y_e = -3{\cdot}226 + 5{\cdot}779 \log z_e, \quad (31)$$

$$Y_m = -2{\cdot}226 + 5{\cdot}779 \log z_m. \quad (32)$$

From the equations, the potency of elliptone relative to that of rotenone is given by

$$M = -0{\cdot}696 \pm 0{\cdot}028, \quad (33)$$

or

$$\rho = 0{\cdot}201 \pm 0{\cdot}013, \quad (34)$$

and the predicted line for the mixture is therefore

$$Y'_m = -1{\cdot}952 + 5{\cdot}779 \log z_m. \quad (35)$$

The median lethal doses are 5·34 mg./l. for rotenone, 26·51 mg./l. for elliptone, and 17·80 mg./l. for the mixture. On the hypothesis of similar action the predicted value of L.D. 50 for the mixture is 15·96; (21) and (22) show that

$$M_m = -0{\cdot}0474 \pm 0{\cdot}0238. \quad (36)$$

The significance of this discrepancy is obtained from the t value of 1·99 (6 degrees of freedom), a value which could be exceeded by chance in about 10 % of cases. This test, therefore, shows no significant evidence of antagonism between rotenone and elliptone, though the mixture was 10·3 % less toxic than predicted from its constituents.

TOXICITY OF A MIXTURE OF ROTENONE AND *l*-α-TOXICAROL

Again using the chrysanthemum aphis as test insect, rotenone, *l*-α-toxicarol, and a mixture containing 9·80 % rotenone were sprayed at various concentrations on 24 Sept. 1941. Application of the usual technique to the probits of the kills obtained showed that

three linear regressions, which did not depart significantly from parallelism, adequately described the data, there being little indication of heterogeneity. Parallel lines were fitted to the probits for rotenone and *l*-α-toxicarol and gave a value of $\rho = 0{\cdot}163$ for the relative toxicity. As in the two preceding sections, the expected probits shown in Table 3 were computed.

TABLE 3. *Summary of results for mixture of rotenone and l-α-toxicarol*

Conc. of mixture mg./l.	Equiv. rotenone mg./l.	No. of insects	%* kill	Observed probit	Expected probits: Independent action	Expected probits: Similar action	w for similar action
5·41	1·32	48	100·0	(7·943)	7·432	7·797	1·190
4·32	1·06	48	100·0	(7·506)	6·825	7·712	1·469
3·25	0·80	49	89·8	6·174	6·094	6·758	9·389
2·16	0·53	50	82·0	5·910	5·065	5·905	23·490
1·09	0·27	50	30·0	4·477	3·494	4·508	29·128

* There were no deaths among the controls.

In the column 'observed probit' the maximal working probit has been inserted for the two cases of 100 % kill. Once again the hypothesis of independent action consistently underestimates the actual kill, and a χ^2 test would demonstrate clearly the significance of this. On the other hand, for the discrepancies between the predictions from similar action and the observed probits

$$\chi^2_{[3]} = 2{\cdot}27, \tag{37}$$

using only the three lowest concentrations, and

$$\chi^2_{[5]} = 2{\cdot}36, \tag{38}$$

if the maximal working probits for the 100 % kills are also included. The joint action of the two constituents of the mixture is thus adequately explained on the hypothesis of similarity.

Parallel regression lines fitted to the three series of probits are

$$Y_r = 7{\cdot}179 + 4{\cdot}657 \log z_r, \tag{39}$$

$$Y_t = 3{\cdot}512 + 4{\cdot}657 \log z_t, \tag{40}$$

$$Y_m = 4{\cdot}263 + 4{\cdot}657 \log z_m, \tag{41}$$

from which

$$M = -0{\cdot}788 \pm 0{\cdot}038, \tag{42}$$

or

$$\rho = 0{\cdot}163 \pm 0{\cdot}014. \tag{43}$$

The *l*-α-toxicarol was thus about one-sixth as toxic as rotenone, and the predicted line for the mixture is

$$Y'_m = 4{\cdot}336 + 4{\cdot}657 \log z_m. \tag{44}$$

From (39)–(41) the median lethal doses for the two pure substances and the mixture were 0·340, 2·087 and 1·440 mg./l. respectively. The relative toxicity of the mixture,

$$\pi_r + \rho\pi_t = 0{\cdot}2451, \tag{45}$$

leads to a prediction of 1·389 mg./l. for the value of L.D. 50 on the hypothesis of similar action. The discrepancy between prediction and measurement is given by

$$M_m = -0{\cdot}0156 \pm 0{\cdot}0321. \tag{46}$$

The mixture was 3·5 % less toxic than similar action predicts, but there is no significant evidence for antagonism.

Data consisting of median lethal doses only

Occasionally it may happen that it is desired to test for synergism data available only in the form of estimates of median lethal doses (or, of course, doses corresponding to any other selected level of kill) for a number of mixtures containing different proportions of two constituent poisons. This will occur if the mixtures have not all been tested at one time; the data can then most conveniently be analysed by reducing the estimated median lethal doses to a comparable basis by the aid of the results from those mixtures tested on more than one occasion.

As an illustration of synergistic action Bliss (1939) takes results obtained by Tattersfield & Martin (1935) on the toxicity of ether extracts of derris root to *Aphis rumicis*. The data are scarcely suitable for this purpose, as they are derived from seven samples of roots of different origin, the composition of the extracts being given simply in terms of rotenone and a dehydro mixture. This latter is known (Martin, private communication) to have varied considerably from root to root in its relative contents of different dehydro compounds. As Bliss has recognized, this fact makes any comparison of the roots based on two constituents only of doubtful value, but, as an example of method, the results are of some interest.

The figures used by Bliss are simply the median lethal doses of seven extracts containing different proportions of rotenone and dehydro mixture. Rejecting one anomalous sample (sample 2), Bliss shows that the data are in agreement with a generalization of (10), and implies that synergistic action is thereby demonstrated. On account of the non-independence of the median lethal doses which he uses, his test is not strictly correct, but the more fundamental error of procedure is that he does not test the possibility that the data may be adequately explained, without recourse to synergism, as resulting from similar action of the constituent poisons. No observations are available on either component applied alone, but similar action would require that an equation of the form (6) should be satisfied by every mixture. It follows that, if Z is the median lethal dose for any mixture,

$$(\pi_r + \rho\pi_d)\, Z = z_r, \tag{47}$$

where z_r is the median lethal dose of rotenone, a constant for all mixtures. This may be written

$$1/Z = (1/z_r)\, \pi_r + (\rho/z_r)\, \pi_d, \tag{48}$$

and the quantities $1/z_r$, ρ/z_r may then be estimated from the regression of $1/Z$ on π_r, π_d. [Note that the form of (48) is such that, in computing sums of squares and products for the estimation of regression coefficients, the usual 'adjustments for the means' must not be applied.]

In Table II of Bliss's paper the values of $\log Z$ are given together with their standard errors. Now

$$\text{S.E.}\,(1/Z) = 2{\cdot}303 \times \text{S.E.}\,(\log Z) \times (1/Z), \tag{49}$$

and the weight, w, to be attached to $1/Z$ is the reciprocal of the square of its standard error. Using S to denote summation over the 6 samples, it is found that

$$\left.\begin{aligned} Sw\,(1/Z^2) &= 3090{\cdot}42, & Sw\,(\pi_r^2) &= 117216, \\ Sw\,(1/Z)\,(\pi_r) &= 18089{\cdot}50, & Sw\,(\pi_r\pi_d) &= 192506, \\ Sw\,(1/Z)\,(\pi_d) &= 35281{\cdot}78, & Sw\,(\pi_d^2) &= 420647, \end{aligned}\right\} \tag{50}$$

whence the estimates are

$$1/Z_r = 0{\cdot}066733, \tag{51}$$

$$\rho/Z_r = 0{\cdot}053335, \tag{52}$$

or

$$Z_r = 15{\cdot}0, \tag{53}$$

$$\rho = 0{\cdot}799. \tag{54}$$

The portion of the sum of squares for $1/Z$ accounted for by the fitting of the two constants is 3088·93, leaving as residual

$$\chi^2_{[4]} = 1{\cdot}50, \tag{55}$$

which measures the discrepancy between the data and the hypothesis of similar action. Clearly there is no contradiction of this hypothesis, and the observations may be completely explained as being due to mixtures of rotenone, of which 15·0 mg./l. will produce a 50 % kill, and dehydro mixture, of which 1 mg. is equivalent to 0·799 mg. of rotenone, acting similarly. There is no necessity to invoke the more complex concepts of synergism, as has been done by Bliss, in order to set up an algebraic model for the behaviour of the rotenone and the dehydro mixture. The median lethal doses given by Bliss and predicted from the chemical constitutions of the extracts are compared in Table 4.

TABLE 4. *Comparison of measured and predicted median lethal doses (data of Tattersfield & Martin, 1935)*

Sample no.	% rotenone equiv.	L.D. 50 (mg./l.)	
		Measured	Predicted
1	57·8	24·9	25·9
3	56·7	25·1	26·4
4	32·8	43·7	45·7
5	32·6	46·3	46·0
6	55·6	25·9	27·0
7	57·9	26·4	25·9

A better example of the application of this method of analysis may be taken from results obtained by Martin (1940) on the toxicity of different derris roots to *Aphis rumicis*. Tests were made on three separate occasions. On the first *Derris malaccensis*, Kinta root (W. 211), *D. malaccensis*, Sarawakensis root (W. 212) and *D. elliptica*, Changi root (W. 214) were compared. Later W. 211, W. 212, and *D. elliptica*, Sarawak creeping root (W. 213) were tested together, and on a third occasion W. 211 and W. 213 were compared with rotenone. The results of the three experiments were used to obtain comparable values of log L.D. 50 for the four roots; these, with their standard errors, are given on p. 282 of Martin's paper.

From analyses of the roots, percentage contents of rotenone, a toxicarol fraction (possibly including sumatrol, etc.) and deguelin concentrate are available. Martin has reduced these percentages to single measures of the rotenone equivalent of the roots, using estimates of the relative toxicities of these constituents from earlier experiments, and has found that these equivalents bear to each other almost the same ratios as the toxicities of the roots. Nevertheless the comparison with rotenone alone, from the third experiment, suggests that there may be some synergism between the constituents of the roots.

The adequacy of the hypothesis of similar action between the constituents may be tested by evaluating the regression coefficients of the reciprocals of the L.D. 50 values on the proportions of the constituents present. It is probably better to leave out the results for rotenone alone, on account of its much greater toxicity, and to test only the internal consistency of the estimates for the four roots.

TABLE 5. *Median lethal doses and constitutions of derris roots*

Root	1/L.D. 50 ($=1/Z$)	Content of: Rotenone (π_r)	Toxicarol (π_t)	Deguelin concentrate (π_d)	Weight (w)
W. 211	$4{\cdot}21\times10^{-3}$	0·0146	0·152	0·086	$68{\cdot}3\times10^{6}$
W. 212	$7{\cdot}40\times10^{-3}$	0·0414	0·043	0·124	$11{\cdot}6\times10^{6}$
W. 213	$5{\cdot}50\times10^{-3}$	0·0346	0·024	0·082	$20{\cdot}5\times10^{6}$
W. 214	$11{\cdot}20\times10^{-3}$	0·0794	0·026	0·122	$2{\cdot}4\times10^{6}$

The material from which the test is to be made is shown in Table 5, the weights to be attached to the observations being obtained from the standard errors of log L.D. 50 as before. To these figures, a regression equation of the form

$$1/Z=(1/z_r)\,\pi_r+(\rho_t/z_r)\,\pi_t+(\rho_d/z_r)\,\pi_d \tag{56}$$

may be fitted. Forming the appropriate sums of squares and products, the regression coefficients are the solutions of the equations

$$\left.\begin{aligned} 74113\,(1/z_r)+\ 194199\,(\rho_t/z_r)+\ 226718\,(\rho_d/z_r)&=13787{\cdot}3,\\ 194199\,(1/z_r)+1612882\,(\rho_t/z_r)+1002626\,(\rho_d/z_r)&=50802{\cdot}5,\\ 226718\,(1/z_r)+1002626\,(\rho_t/z_r)+\ 857072\,(\rho_d/z_r)&=47897{\cdot}7. \end{aligned}\right\} \tag{57}$$

The solutions are

$$1/z_r=0{\cdot}102017, \tag{58}$$

$$\rho_t/z_r=0{\cdot}004582, \tag{59}$$

$$\rho_d/z_r=0{\cdot}023539. \tag{60}$$

Now

$$Sw\,(1/Z)^2=2766{\cdot}95, \tag{61}$$

and the portion of this sum of squares accounted for by the regression on the three variables is

$$(1/z_r)\,Sw\pi_r\,(1/Z)+(\rho_t/z_r)\,Sw\pi_t\,(1/Z)+(\rho_d/z_r)\,Sw\pi_d\,(1/Z)=2766{\cdot}78. \tag{62}$$

The remainder gives a test of goodness of fit with

$$\chi^2_{[1]}=0{\cdot}17, \tag{63}$$

a value which could easily be exceeded as a result of chance variations. This test may be a little in error on account of the non-independence of the estimates of log L.D. 50 (and of their standard errors) for the four roots. But this error could scarcely disturb the very clear conclusion that the median lethal doses of the roots are in no way inconsistent with the hypothesis that their three constituents act similarly. From (58) an estimate of the L.D. 50 for rotenone alone is 9·80 mg./l.; (59) and (60) show the toxicities of the toxicarol fraction and the deguelin concentrate relative to this to be 0·045 and 0·231 respectively. These may be compared with the figures of 0·067 and 0·200 used by Martin in computing rotenone equivalents for the four roots. In Table 6 the values of L.D. 50 given in his paper are compared with predictions from the chemical analyses of the roots derived from (56).

TABLE 6. *Comparison of measured and predicted median lethal doses (data of Martin, 1940)*

Root	% rotenone equivalent	L.D. 50 (mg./l.): Measured	Predicted
W. 211	4·13	237·5	237·5
W. 212	7·19	135·1	136·3
W. 213	5·46	181·9	179·5
W. 214	10·87	89·3	90·2

It is clear that, over the range of proportionate constitutions covered by the four roots, there is no evidence of synergistic action. On the other hand, Martin has suggested that the results for rotenone alone in the third experiment, when compared with the rotenone equivalent of W. 213, may indicate synergism. The basing of this conclusion on W. 213 is in some ways undesirable, as the behaviour of this root is rather different in the two experiments in which it occurs. Using instead W. 211, the rotenone equivalent found from Table 4 of Martin's paper is 5·90 % as compared with 4·13 % estimated from the chemical constitution. The enhanced value given by the direct test may be due to synergism, which must then presumably have occurred to much the same extent in all the roots, but it is not easy to judge the significance of the effect. Data on a greater number of mixtures or roots would be needed in order that this point might be investigated more thoroughly.

The method of this section is undoubtedly open to criticism, though most of the criticisms would apply with equal force to the corresponding tests suggested by Bliss. In all cases where the techniques of preceding sections can be applied, they are to be preferred, and this last method reserved for cases in which the median lethal doses provide the only level of comparison for the mixtures tested.

Summary

Mathematical formulae proposed by Bliss for the description of possible modes of action of mixtures of poisons have been discussed. His suggested representation of synergistic action is considered to be inadequate and to have no logical relationship with the simpler concept of similar action. An alternative has been presented, but no data have been found on which to test it.

Three toxicity tests, on mixtures of rotenone with deguelin concentrate, elliptone, and *l*-α-toxicarol respectively, have been examined. In each case, a hypothesis of independent action of the constituents would underestimate the toxicity of the mixture, but similar action satisfactorily predicts the observed percentage kills. Statistical tests for the assessment of the significance of departures from the hypotheses have been developed and applied. When similar action adequately describes the effects of poison mixtures, the potency of any such poison is the same as that of its rotenone equivalent, obtained by addition of the rotenone dosages equivalent to the amounts of the separate constituents present.

A third statistical method for the investigation of similar action has been devised. By its use, data on the toxicity of ether extracts of various derris roots, which have been used by Bliss as an example of synergistic action, have been shown to be perfectly consistent with the simpler hypothesis of similar action between the rotenone and dehydro mixture contained in the extracts. A further series of results by Martin on extracts of four derris roots have been tested in the same way, and shown to be consistent with a hypothesis of similar action between the quantities of rotenone, toxicarol, and deguelin concentrate estimated from chemical analyses. The comparison of these roots with rotenone alone does, however, suggest that some synergistic action may have occurred.

The origin of this paper lies in frequent discussions with Dr J. T. Martin of the Insecticides Department, Rothamsted Experimental Station. For these promptings, and for permission to use his extensive data, I should like to express my gratitude.

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TRANSMISSION OF POTATO VIRUS *Y* BY *APHIS RHAMNI* (BOYER)

By B. KASSANIS, Ph.D.
Rothamsted Experimental Station, Harpenden, Herts

Although *Aphis rhamni* (Buckthorn aphis) is one of the commonest aphides found on potatoes and is sometimes sufficiently abundant to cause direct leaf injury, there is no record of it acting as a vector of a potato virus in Britain. Schultz & Folsom (1925) in America showed that it transmitted mild mosaic, a disease since found to be caused by potato virus *A*. As it readily transmits severe etch virus (Kassanis, 1941), and this virus has relationships with its vectors similar to those of potato virus *Y*, it seemed possible that it might also transmit virus *Y*. Both viruses belong to the group called non-persistent by Watson & Roberts (1940); vectors of such viruses soon lose their ability to transmit, and their efficiency is enhanced if they are starved before feeding on the source of infection and if the infection feeding is restricted to a few minutes.

The *A. rhamni* used in this work were identified and supplied by Mr J. P. Doncaster; they were kept on either healthy potato plants or the winter host, *Rhamnus cathartica*. In each test transmissions were also made with *Myzus persicae*, so that the relative efficiencies of the two aphides as vectors could be compared. All the insects were allowed to feed on the source of infection for from 2 to 5 min., when they were transferred immediately to the test plants. Some of the insects were starved for 4 hr. before getting their infection feeding, and the others were taken from healthy plants on which they had been feeding continuously. Infected tobacco, var. White Burley, and potato, var. Majestic, were used for the infection feedings and healthy plants of these for test plants. The results are summarized in the table. They show that in the glasshouse at least *Aphis rhamni* is as efficient a vector of potato virus *Y* as *Myzus persicae*, and that it reacts to a preliminary starving period in the same way.

Transmission	From tobacco to tobacco	From potato to potato
No. of insects used per plant ...	5	10
M. persicae, starved	15/15*	5/5
A. rhamni, starved	22/25	7/10
M. persicae, unstarved	7/15	0/5
A. rhamni, unstarved	7/20	3/10

* The denominator is the number of test plants used and the numerator the number that became infected.

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REVIEWS

Diseases of British grasses and herbage legumes. By KATHLEEN SAMPSON and J. H. WESTERN. Pp. 85. Cambridge: at the University Press. 1941. 5*s.* 0*d.*

This bulletin, which is issued for the authors by the British Mycological Society, gives a clear and succinct account of the mycology, with notes on the symptoms and control, of diseases of grasses (smuts, rusts, mildew, leaf-spot diseases, inflorescence diseases, and systemic infection of *Lolium*), turf (seedling diseases, diseases of established turf, control of turf diseases, and fairy rings), herbage legumes (root and crown diseases, miscellaneous root diseases, stem diseases, leaf-spot diseases, mildews, rusts, anther mould, virus diseases, and diseases of unknown origin), and mineral deficiency diseases of grasses and herbage legumes. The bulletin is well produced, is illustrated by eight excellent plates and fifteen text-figures, contains a useful selected bibliography of 315 references, and terminates with a good index. More critical attention might have been given to the method of stating the dimensions of conidiophores and spores, for whatever these are worth, and in many cases there is discrepancy between the dimensions given in the text and those shown in the illustrations. In Text-fig. 2 the ascus figured could hardly be described as 'biseriate', in Text-fig. 12 one of the 'non-septate' conidiophores is apparently septate, and in Text-fig. 15 the scale is inaccurate by the magnification stated. On p. 25 distinction is made between 0·6 and 0·7 μ, whereas on p. 40 certain structures are described as 'as big as a pea, or even larger'! Although the work is, perhaps, too incomplete for research purposes it will be extremely convenient for reference and student use. W. B. BRIERLEY.

A handbook of home-grown timbers. D.S.I.R. Forest Products Research. Pp. iv+68. London: H.M. Stationery Office. 1941. 1*s.* 6*d.*

A third and, alas! very much 'War Emergency edition' of this extremely useful and well-written work which has been brought thoroughly up to date. It contains a remarkable amount of information of immediate importance to anyone interested in trees and timber and, even in its new pamphlet form, is excellent value for money. W. B. BRIERLEY.

A revision of Melanconis, Pseudovalsa, Prosthecium, *and* Titania. By L. E. WEHMEYER. Pp. viii+161, plates 11. Ann Arbor, U.S.A.: University of Michigan Press. 1941. $2.50.

This is a companion volume to the author's *The Genus* Diaporthe *and its Segregates*, published in 1933. In addition to the four genera named in the title an appendix contains a revision of the genera *Calospora* (which is not considered valid), *Massaria*, and *Aglaospora*, to which many species of the main genera are transferred. Characteristically all the species considered are found on hardwood trees and shrubs where, in certain cases, they may cause unimportant diebacks. Prof. Wehmeyer is, however, not interested here in the phytopathological problems associated with these fungi but writes purely as a systematic mycologist. His work is a model of taxonomic study and the volume has been produced in a manner worthy of its scientific value. W. B. BRIERLEY.

Further studies on cereal rusts in India. By K. C. MEHTA. Pp. vii+224. Scientific monograph No. 14, Imperial Council of Agricultural Research. Calcutta: Government of India Press. 1940. 9*s.* 6*d.*

This volume contains an account of a series of investigations which have extended over about sixteen years and which are here described together instead of being published at intervals in scientific journals. The account falls into three portions: Sect. I deals with physiological races especially of *Puccinia graminis tritici* and *P. graminis avenae*, *P. triticina*, and *P. glumarum*; Sect. II deals with the role of *Berberis* and *Thalictrum* as alternate hosts; Sect. III deals with oversummering in relation to annual recurrence. The work is a notable contribution to our knowledge of the types, distribution, and behaviour of the physiological races of cereal rusts in India, but as a scientific publication the volume gives my editorial soul greatly to writhe! W. B. BRIERLEY.